# OPTIMIZATION OF *IN VITRO* MAMMALIAN BLASTOCYST DEVELOPMENT: ASSESSMENT OF CULTURE CONDITIONS, OVARIAN STIMULATION AND EXPERIMENTAL MICRO-MANIPULATION

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Factors currently at the forefront of human in vitro fertilization (IVF) that collectively influence treatment success in the form of blastocysts development were investigated during early mammalian embryology with concentration on infertile patients presenting with diminished ovarian reserve or preliminary ovarian failure. A novel experimental technique, graft transplant-embryonic stem cells (GT-ESC) was introduced in the mouse model, as the first inclusive approach for embryo selection in IVF treatments resulting in successful graft integration of sibling cells, stage-dependent (day 4) blastocysts. E-Cadherin-catenin bonds play an integral role in trophectoderm cell viability and calcium removal, inducing disruption of cell-to-cell bonds at the blastocyst stage was detrimental to continued blastocyst development. One of the leading methods for embryo selection for uterine transfer in human IVF is application of pre-implantation genetic screening (PGS) methods such as next generation sequencing (NGS). Female patients <35 y do not benefit from this treatment when outcome is measured by presence of fetal heart beats at 10 weeks of gestation. Patients 35-37 y benefit from PGS with no significant difference of outcome based on form of PGS method utilized. Therefore, small nucleotide polymorphism array (snp-array) or targeted-NGS should be selected for this age range to lessen the financial burden of the patient. Embryos from women >40 y have a higher rate of mosaic cell lines which can be detected by NGS. Therefore NGS is most beneficial for women >40 y. Additionally, ovarian stimulation of the patient during human IVF can notably influence outcome. Anti-Müllerian hormone (AMH) is a more conducive indicator of blastocysts development per treatment compared to basal follicle stimulating hormone (FSH). Actionable

variables included in a decision tree analysis determined a negative influence (0% success, n=11) of high dose gonadotropin use (>3325 IUs) in good prognosis patients (>12 mature follicles at trigger, AMH >3.15 ng/mL). A positive relationship exists (80% success, n=11) between poor responders (AMH <1.78 ng/mL, <12 mature follicles at trigger) and high dose gonadotropin use (>3025 IUs). Utilizing the decision tree during IVF treatment can be beneficial to treatment success. Moreover, a parallel relationship of the fundamental principles of culture medium pH, pCO2 and pO2 was found with respect to blastocyst development. Human infertility patients' gametes predisposed to primary stressors (i.e., age, genetics and etiology) are negatively impacted (~30% success, n=7) for cleavage stage (day 3) embryo development when primary culture medium has pCO2 <30mmHg given age >31 y and <14 oocytes retrieved. When day 3 embryo development is measured at >65% good quality embryos per treatment (based on SART grading criteria), blastocysts development success is highest when secondary culture medium pO2 is 69-88 mmHg (~90% success, n=12). Thus, IVF treatment outcome can be optimized with utilization of predictive model analyses in the form of decision trees providing greater success for the IVF laboratories, ultimately decreasing the emotional and financial burden to infertility patients.

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| | |
|---|---|
| aCGH | Array comparative genomic hybridization |
| AMH | Anti-Müllerian hormone |
| bHCG | Beta human chorionic gonadotropin hormone |
| CoA | Certificate of analysis |
| DET | Double embryo transfer |
| DOR | Diminished ovarian reserve |
| E2 | Estradiol |
| ET | Embryo transfer |
| FET | Frozen embryo transfer |
| FHB | Fetal heartbeat |
| FISH | Fluorescent *in situ* hybridization |
| FSH | Follicle stimulating hormone |
| GLM | Generalized linear model |
| GnRH | Gonadotropin releasing hormone |
| GPGD | Calcium/magnesium free solution |
| HCG | Human chorionic gonadotropin |
| HIF | Hypoxia inducible factor |
| HIPAA | Health Information Portability and Accountability Act |
| hMG | Human menopausal gonadotropin |
| ICM | Inner cell mass |
| IU | International units |
| IU | International units |
| IVF | *In vitro* fertilization |
| mHTF | Modified human tubal fluid |
| NGS | Next generation sequencing |
| PBS | Phosphate buffered saline |
| PGS | Preimplantation genetic screening |
| POF | Preliminary ovarian failure |
| QA | Quality assurance |
| QC | Quality control |

| | |
|---|---|
| SART | Society of American Reproductive Technology |
| SET | Single embryo transfer |
| SNP array | Single nucleotide polymorphism array |
| TE | Trophectoderm |
| TVOR | Transvaginal oocyte retrieval |

# CHAPTER 1

# INTRODUCTION

## 1.1 Central Theme/Background

Conception occurs naturally for many women. However, for many others *in vitro* fertilization (IVF) may be required. An average of 7.3 million women in the United States have used infertility services reported to the centers of disease control (CDC), which is 7.3% of married women aged 15-44y ("FastStats" 2017). During a menstrual cycle that is regulated by hormones, such as follicle stimulating hormone, luteinizing hormone, estradiol and progesterone, a follicle matures and the mature oocyte is released from the ovary. Upon ovulation, the oocyte is fertilized in the fallopian tube and the resulting embryo continues to divide and implant in the uterus, creating a pregnancy. The embryo develops into a placenta and a fetus, ultimately leading to a live born infant. When there are problems or complications associated with the reproductive health of women, assisted reproductive technology services (ART) are often used. Some examples of such complications include advanced maternal age (related to diminished ovarian reserve), premature ovarian failure (POF) and poly-cystic ovary disease. One of the solutions that ART offers is *in vitro* fertilization (IVF). In this process, many of the complications mentioned above are overcome by the use of controlled ovarian hyperstimulation which allows for the development of multiple embryos. This leads to excess embryo development for some women while others experience low to no embryo development. Two of the leading conditions for the latter group of patients involve women with diminished ovarian reserve or preliminary ovarian failure.

Diminished ovarian reserve (DOR) is a condition characterized by poor fertility outcomes and currently presents as a major challenge in reproductive medicine, particularly *in vitro*

fertilization (IVF) (Cohen et al. 2015; Atasever et al. 2016; Levi et al. 2001). Women are diagnosed with DOR if they demonstrate at least one of the following:

- High follicle stimulating hormone (FSH) levels when not in the follicular phase
- Low antral follicle counts
- Low anti-Müllerian hormone (AMH)

When diagnostic testing is performed to aid in the diagnosis and treatment of women who wish to bear children, routine tests including serum hormone assays and ovarian ultrasounds are performed. When a woman is in her reproductive years with good prognosis, her FSH is expected to be between 3.1-7.9 mIU/ml (Atasever et al. 2016; Ryley et al. 2005; Abdalla 2004). AMH is used to assess the remaining ovarian reserve of a female as well as a predictor of her response to controlled hyperstimulation of the ovaries during IVF treatment and her AMH is expected to be ≥1 ng/ml.

Diminished ovarian reserve patients often use assisted reproductive technology to increase their chances of a successful pregnancy. The Center for Disease Control's (CDC) national assisted reproductive technology surveillance system report for 2013 confirms US clinics performed 190,773 IVF procedures that year. A remarkable 32% of patients within this group were diagnosed with DOR (National ART Summary Report for 2013.). This subgroup of patients seeking infertility treatments are composed of women that have advanced maternal age, defined as 38 years or older or premature ovarian failure (POF). Premature ovarian failure is defined as a condition in which a woman's ovaries enter early menopause due to amenorrhea, hypergonadotropism and hypoestrogenism or genetic reasons. One such genetic disorder is fragile-X, which is a single gene disorder that can cause 20% of women who are carriers to enter early menopause before the age of 40 (Santoro et al. 2012; Saldarriaga et al. 2014.; Chapman et al. 2015). In today's society,

women are more active and valuable in the work force. As a result, families opt to delay child bearing until they are satisfied in their career achievements. With female age, fertility declines. IVF, the solution to the increasing rate of infertility is not perfect. Some of its problems are now discussed.

## 1.2 Statement of the Problem

IVF is the primary treatment offered to patients of advanced maternal age and diminished ovarian reserve. IVF is preceded by controlled hyperstimulation of the ovaries by the use of recombinant gonadotropins such as FSH and luteinizing hormone (LH). These are the two main stimulatory agents used by most reproductive endocrinologists in the United States (Pouwer et al. 2015; Muasher et al. 1988; Farquhar et al. 2013). The leading benefit of controlled ovarian hyperstimulation is the production and retrieval of many oocytes per IVF treatment with an opportunity of increased pregnancy rates due to the creation of multiple embryos. Yet, IVF has not reached a high efficiency when considering the number of oocytes retrieved and the resulting high quality implantable blastocysts that develop from them. This is one of the major problems in the IVF industry and the proposed studies here give valuable information on improved culture conditions leading to higher pregnancy rates as well as introduction of a micromanipulation technique that could possibly lead to reduced embryo discard rate.

Many more oocytes are produced per IVF treatment and fertilized than the number of children the family intends to have. However, currently roughly 60% of fertilized oocytes develop into blastocysts, which are defined as embryos at the $5^{th}$ day of development and have two differentiated cell types, (discussed in Embryo Development section). Approximately half may be

suitable for uterine transfer or cryopreservation, defined as high quality blastocysts[1] (Zhao et al. 2012; Costa-Borges et al. 2016). Often, IVF treatments for women over the age of 35 years, embryos are observed to be of poor quality in either one of the cell types. Meanwhile, the other cell type may be fair to good quality. Embryos that demonstrate this form of "mixed quality" development are discarded due to poor grading per standard operating protocol in IVF laboratories. In patients that are characterized as DOR, their number of oocytes produced per IVF treatment is usually lower than 8, with only 80% expected to be mature for fertilization (Hariton et al. 2017; Broer et al. 2011; Lai et al. 2013). By day 5 post fertilization just 2-3 may develop into blastocysts. Due to the poor prognosis of this group of infertility patients, often times the few blastocysts that develop are not suitable for uterine transfer or cryopreservation, rendering the IVF treatment a failed attempt. To propose a solution, the underlying DOR and POF conditions as well as embryonic development should be well understood and is discussed in the next sections.

## 1.3 Review of Literature

IVF success relies on many aspects of developmental and genetic biology. Some of the many important aspects are infertility conditions such as diminished ovarian reserve and preliminary ovarian failure as well as developmental aspects such as *in vitro* culture conditions, embryonic cell differentiation, and genetic anomalies such chimeric growth. The following sections review many of the factors that contribute to the success of IVF treatments for infertility patients.

[1]. High quality blastocyst is a common term that is used by embryologists to signify that an embryo has been graded in a subjective manner and determined to be of high implantation potential based solely on its morphology at the time of grading. Different grading systems are used by laboratories. The grading system used in my studies is the SART Grading System (Racowsky et al. 2010).

### 1.3.1 Embryo Development

An embryo is created when a sperm cell successfully fertilizes an oocyte. This event marks the beginning of embryo development referenced as day 1. Upon fertilization, pronuclei appear and are visible between 16-19 hours post fertilization the first cell differentiation occurs by day 4 of embryonic development. This is the stage where a ≥8 cell embryo forms a morula, a compacted mass of cells, an event unique to mammalian embryo development (Piccolomini et al. 2016; Nikas et al. 1996; Pera and Prezzoto 2016). The cells that are compacted on the inner surface of that sphere then differentiate into inner cell mass. The cells that are touching the inner cells on one side of their surface and have no neighboring cells on the other side, then develop into *trophoblast* or *trophectoderm* cells (Pera and Prezzoto 2016). Once the compaction is complete, the embryo then begins to collect fluid from its environment and a cavity called a *blastocoel* begins to form as seen in figure 1.1a. This blastocoel continues to grow in size as the embryo collects more fluid.

Approximately 5 days post fertilization, human embryos (called “blastocysts” at this stage) have differentiated in two cell types: inner cell mass (ICM) and trophectoderm (TE) (Ivec et al. 2011; Mihajlović et al. 2015; Bavister 2012). This is the first binary differentiation in mammalian development. Prior to blastocyst formation, each embryonic blastomere expresses both the Cdx2 and Oct4 transcriptional factors, which allow the cell to become trophoblast or ICM cells (Gilbert 2014). Upon differentiation, the cell expresses a set of genes specific to each region. Only trophoblast cells make Cdx2 and down regulate Nanog and Oct4 transcriptional factors. Cdx2 is only synthesized in the trophoblast cells forcing the down-regulation of Oct 4 and Nanog. Cdx2 activation is regulated by Yap protein which in turn is a co-factor for the transcription co-factor Tead4. Phosphorylated Yap cannot enter the nucleus and is degraded. The ICM cells Tead4 cannot function and Cdx2 remains untranscribed. Cdx2 blocks the expression of Oct4 and Oct4 blocks

the expression of Cdx2.In this way, the two cell types remain differentiated. This binary differentiation is a result of ICM cells are the group of cells that form the fetus, while the TE cells mainly develop into a placenta and cord (figure 1.1b). On day 6 of embryo development, the TE cells stretch while expanding and the zona pellucida that encompasses the embryo thins and tears allowing for hatching of the embryo (Lee et al. 2016). Once completely hatched, the embryo will come into contact with the uterus and the process of implantation will start (Ruane et al. 2017; Teh et al. 2016; Watson 1992), discussed in the next section.

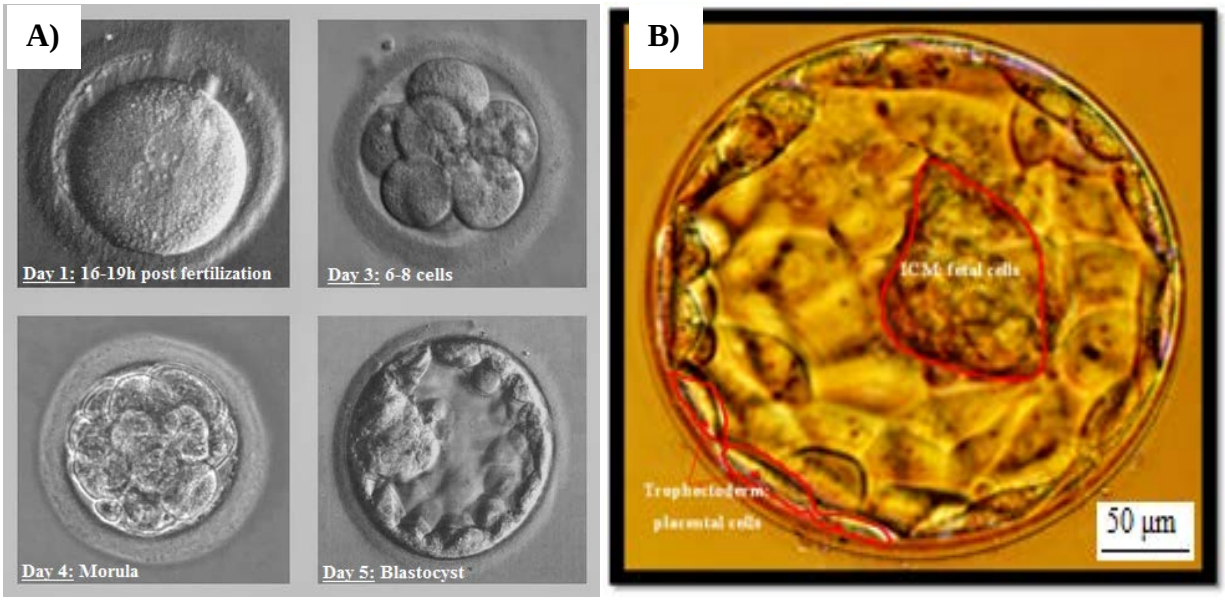



Figure 1.1. Human embryo development. A). On day 1, fertilization can be confirmed by the two visible pronuclei. On day 3 of development, the embryo has divided into 6-8 cells. By day 4, cell division continues and the cells compact to form a morula. On day 5, the embryo has cavitated and a cavity called a blastocoel has been created. Cells have differentiated into inner cell mass and trophectoderm cells. Courtesy: Dallas IVF. B). Human blastocyst. This blastocyst is at 5 days post fertilization. The diameter is approximately 250µm. The blastocyst is completely expanded containing a fluid filled blastocoel and the inner cell mass (ICM), outlined in red, is easily distinguishable from the trophectoderm (TE) cells. Three of the TE cells are traced in red against the zona pellucida. Courtesy: Dallas IVF

### 1.3.2 Placenta and Fetus

On day 5 of development, an embryo begins the process of implantation in the uterus. The first step of this process is called *apposition,* which is defined by the embryo embedding into the

endometrium (Ruane et al. 2017; Teh 2016). Ruane et al. (2017) went on to state that during this step, between days 5-8 of development, the trophoblast cells continue to multiply and during the process evade into the endometrial tissue. The complete evasion of the cells into the endometrium is called *adhesion*. The endometrial tissue grows over the developing embryo to completely coat it. At the same time, the pools of blood, supplied by the maternal vessels seen in figure 1.2, in the uterine lining that are being supported by blood vessels in the uterus begin to coalesce. The trophoblast cells are continuing to multiply and some of them between days 7-12 of development, fuse together to form large sheaths of multinucleated cells called the *syncytiotrophoblasts*. Between days 12-18, these sheaths continue to grow and extend into the endometrium to make fingerlike projections called the *villi*. The trophoblast cells that have not participated in the fusion are now called *cytotrophoblasts*. Inside the villi the fetal blood vessels develop (Evans et al. 2016). By day 23, the pools of blood in the uterus have fused together in between the villi and are in close contact, but not fused together due to the membrane of trophoblast in between them. It is now that the nutrients from the uterine blood can diffuse into the fetal blood and the waste products from the fetal blood can diffuse out to the uterine blood (Zohni et al. 2016; Carter and Enders 2016). The structure continues to grow during the pregnancy, to a point where it lines almost the entire depth of the uterus and as it grows, more nutrients can be diffused across. This structure matures into a *placenta* (Atlas de Reproducción Asistida 2014).

The placenta has many functions. Mainly, it ensures the adequate blood supply for the fetal-placental unit to have exchange of nutrients, oxygen and wastes between the mother and the fetus (Acharya et al. 2016). It also protects the developing fetus from the maternal immune system attack. The placenta anchors the embryonic and extra embryonic structures of the blastocyst to the uterine wall and releases hormones such as progesterone, estrogen, and chorionic gonadotropin

(hCG) (Handwerger and Freemark 2000). These hormones, amongst others, are essential to fetal life. Progesterone supports embryo implantation by stimulating an increased amount of human chorionic gonadotropin (hGC) secretions necessary for fetal nutrition. Human chorionic gonadotropin is a protein hormone that is similar in structure to the luteinizing hormone.

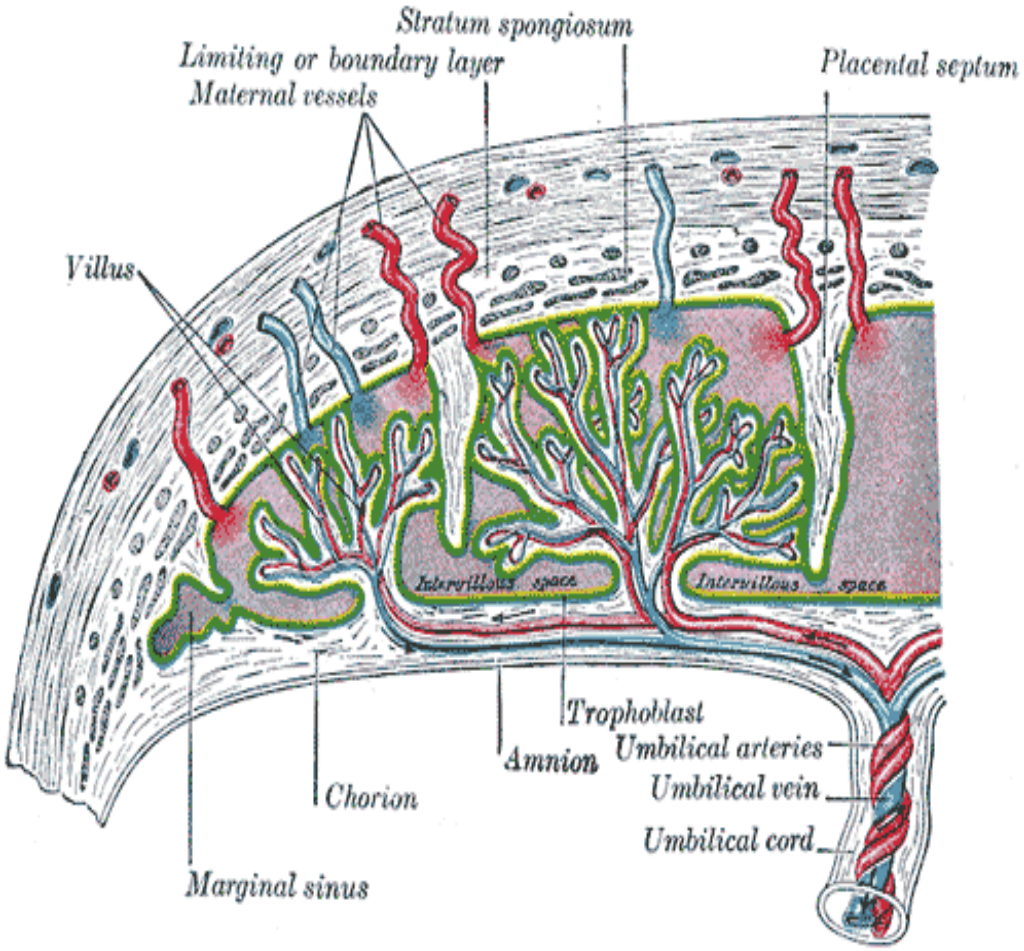



Figure 1.2. Placental structure. The organization for the blood vessels in the placenta can be seen here. The maternal vessels provide a pool of nutrient rich oxygenated blood to surround the villus, which carries fetal blood. The thin layer of cells separating the mother's blood from fetal blood is formed from the trophectoderm cells. Source: Henry Vandyke Carter - Henry Gray (1918) *Anatomy of the Human Body*.

In the early pregnancy its primary role is to keep the corpus luteum functioning so it can continue to produce estrogen and progesterone (Kayisli et al. 2003). Later in pregnancy, it may have an anti-body effect, which is thought to protect the fetus from being rejected by the mother's body. It

is also the hormone that allows the testes to grow by the production of testosterone. Elevated levels of progesterone are necessary to prevent spontaneous abortion and contractions of the uterus (Andersen et al. 2016). Estrogen is crucial for the process of proliferation (Evans et al. 2016). This study went on to state that estrogen is responsible for the enlargement of the uterus to allow growth of the fetus and the enlargement of breasts for the production of milk. Elevated levels of estrogen will also increase blood supply towards the end of pregnancy through a process called *vasodilatation* (Corcoran et al. 2014; Wang and Zhao 2010).

The placenta connects to the fetus by the umbilical cord through the abdomen (figure 1.3).

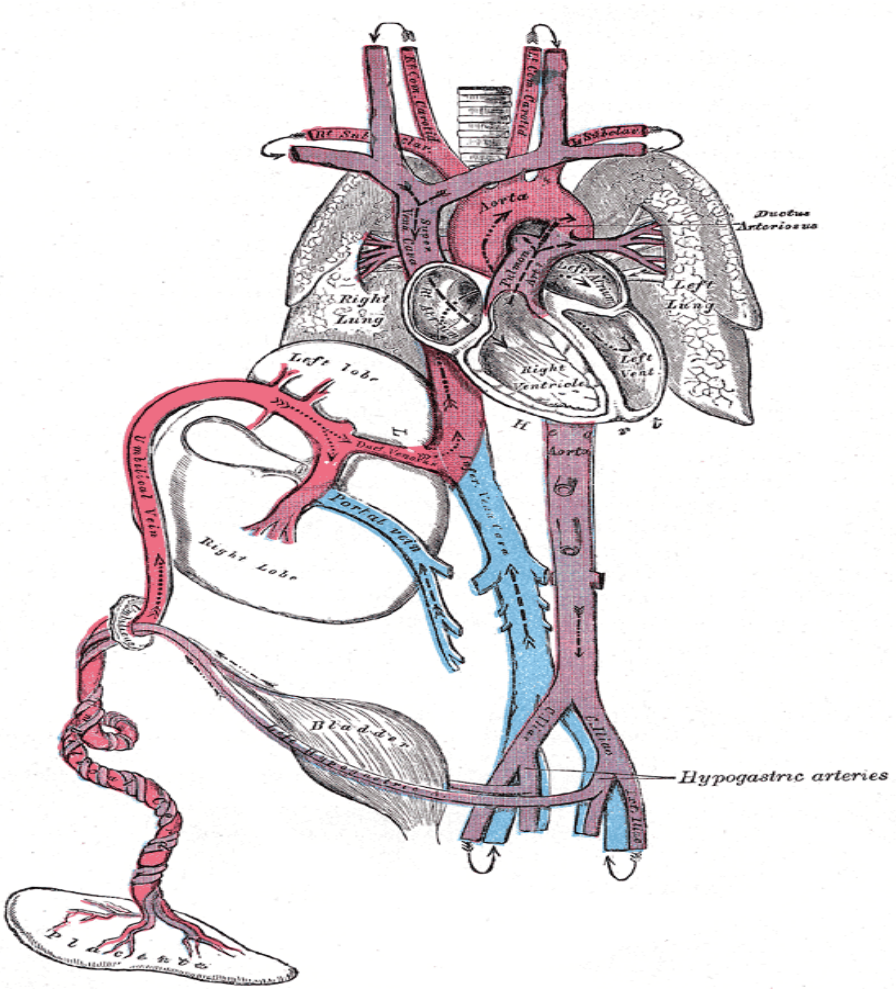



Figure 1.3. Fetal circulation plan. One of the branches joins with the hepatic portal vein connecting it to its left branch carrying blood to the liver. The *ductus venosus*, second branch bypasses the liver to flow into the inferior vena cava, which carries fetal blood towards the heart. Source: *Anatomy of the Human Body*, 2000

The cord has an umbilical vein that continues towards the liver where it splits into two branches. One of the branches joins with the hepatic portal vein connecting it to its left branch carrying blood to the liver. The second branch is known as the *ductus venosus* and bypasses the liver to flow into the inferior vena cava, which carries fetal blood towards the heart. There are two umbilical arteries that branch from the internal iliac arteries and pass on each side of the bladder back into the umbilical cord to complete the circulation into the placenta (Gray 2000; Wang and Zhao 2010). It is clear that this is where the placenta and the fetus interact making this the critical developmental aspect of the growing embryo that may cause complications with the success of the novel technique introduced in this research (Chapter 3*)*.

Mentioned earlier, some women experience infertility. Two of the conditions that lead to infertility are diminished ovarian reserve and preliminary ovarian failure. These conditions are now discussed.

### 1.3.3 Diminished Ovarian Reserve

Women with true diminished ovarian reserve are a large part of the IVF programs in the United States ("National ART Summary Report for 2013" 2015; Cohen et al. 2015). Many fertility centers will often refuse treatment to this group of patients with their own oocytes due to the extremely poor prognosis (Lamazou et al. 2012; Ethics Committee of American Society for Reproductive Medicine 2012). There are smaller IVF programs that will offer a reduced multi-IVF treatment program to this group of patients, which financially helps the patient and the IVF center (Mathews et al. 2009; Ben-Rafael and Feldberg 1993). On the other hand, multiple treatments of injectable gonadotropins and a hyperstimulated, super physiological state of the female patient places her at risk for thrombosis and ovarian hyperstimulation syndrome amongst

other things (Sousa et al. 2015). Even with multiple treatments the programs can only offer a small insignificant number of pregnancies and live birth in this category (Vega et al. 2016; Ben-Rafael and Feldberg 1993; Oehninger 2011).

Preliminary ovarian failure (POF) is one of the conditions that lead to DOR (Alborzi et al. 2015). POF represents a condition, which is characterized by the absence of normal ovarian function due to the depletion of primordial follicles before the age of 40 years (Butts et al. 2013). With this condition women are reported to have amenorrhea. Other symptoms usually include arrhythmia, intolerance and hot flashes (Broekmans et al. 2009; Butts et al. 2013). An accurate clinical diagnosis can be made if there is an absence of menses for at least 4 months in combination with FSH levels exceeding 40 IU/L before the age of 40 years (Safdarian et al. 2012).

The aging ovary goes through a series of hypothalamic-pituitary-ovarian axis irregularity. This is a negative feedback system of pulsatile gonadotropins secretion. Studies in postmenopausal women compared with premenopausal women have shown elevated LH and FSH levels and increased LH pulse amplitudes without a change in LH frequency (Gill et al. 2002; Barroso et al. 2001). This altered feedback of aging ovaries may be due to a lack of steroid priming and the result of ovarian factors. With elevating FSH levels, a higher number of antral follicles are grown to dominant follicles along with a larger cohort of recruitment per menstrual cycle. This is the leading reason for dizigotic twinning as well as early stages of menopause. With each passing menstrual cycle the number or remaining follicles are lessened and women presenting with DOR or POF are then facing infertility. With a correct diagnosis, the option of IVF is usually pursued. According to the Society of Assisted Reproductive Technology (SART), the most current data, which includes live births is published for 2014. A total of 190,384 treatments were performed that year. Figure 1.4 reflects the percentage of treatments resulting in live births per age category with an average

of only 23% of embryo transfers resulting in viable babies delivered. This data reports only those treatments resulting in viable embryos for transfer. Treatments in which embryos were not transferred due to no blastocyst development, oocyte banking, embryo banking or complete arrest in development are not reported. Furthermore, it is clearly observed here that there is a steep decline in success rates after the age of 40 years. The patients that have the least chances of success are in the age category of greater than 42 years with success of 4% per IVF treatment. The only method to improve this outcome currently is with the use of donor oocytes.

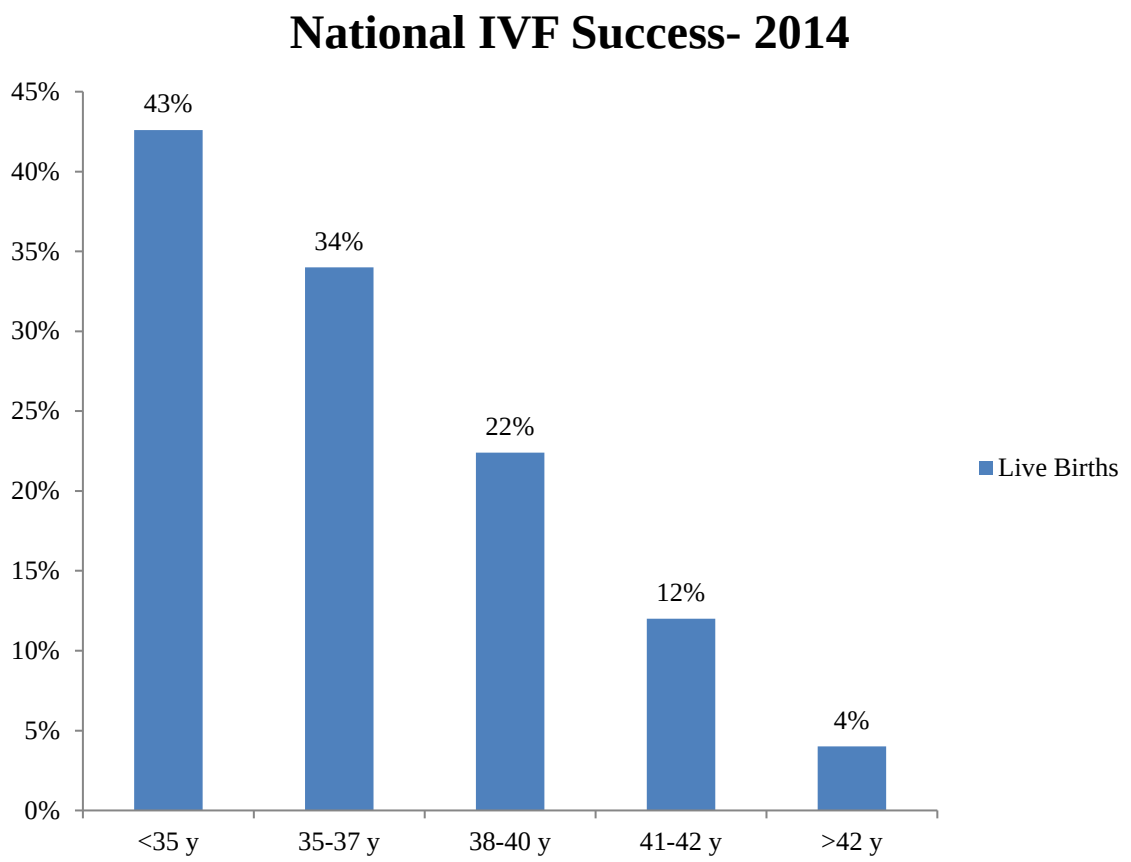



Figure 1.4. National IVF success rates for 2014. A total of 190,384 treatments were performed that year. The figure reflects the percentage of treatments resulting in live births per age category, stating that average of only 23% of embryo transfers resulted in viable babies delivered.
Source: https://www.sartcorsonline.com/rptCSR_PublicMultYear.aspx?ClinicPKID=0

As the culture across the globe evolves towards a more career driven society for women, each year the age of child bearing is later. The average age of first-time mothers increased 3.6

years from 1970 to 2006 according to the data collected by the centers of disease control (Mathews et al. 2009). With the accepted use of contraceptives and awareness of timed treatments, unplanned pregnancies occur less often. There can be benefits to delaying child-bearing; however it can make it more difficult for a woman with increased age to conceive naturally as age can affect fertility, pregnancy and childbirth. A woman's fertility begins to decline at a much faster rate at 36 years of age and varies from woman to woman (Hart 2016; Meldrum et al. 2016; Broekmans et al. 2009). Currently there is no guaranteed method that will estimate when this will be for a particular woman or any symptoms that arise to warm the onset of this decline.

The gradual change in fertility is due to a decrease in the number and quality of oocytes remaining in the ovaries (Sauer 2015). As the age increases so does follicle stimulating hormone (FSH) levels causing more antral follicles to grow and arrest per menstrual cycle than a woman with a normal range FSH level. This causes the higher rate of declining ovarian reserve (Cohen et al. 2015). This event is well correlated to the decline in the quality of oocytes remaining. Increased female age will result in a lowered percent of chromosomally balanced oocytes, increasing the chances of early miscarriage or a pregnancy with an aneuploid fetus (Stoop et al. 2014; Shahine et al. 2016; Staessen et al. 2008). Also associated with increased age is the increased risk of ectopic pregnancy, preeclampsia, hypertension, gestation diabetes, placental complications, intrauterine growth restriction, and birth by cesarean section. Babies born may have lower birth weight and non-genetic malformation (Reproductive Endocrinology and Infertility Committee 2011).

Fertility treatments are desired mostly by patients that are unsuccessful at achieving a pregnancy within the first 6 months of trying to conceive when under 35 years of age and within the first 12 months when over the age of 35 years (Aghajanova et al. 2016; Gnoth et al. 2003). Medical treatments cannot make up for the age related fertility decline, however can offer

treatment that increases the chances of a successful pregnancy by the use of assisted reproductive technology (ART). Diagnosis are formulated with the combination of female partner's age, medical history, sperm parameters, uterine lining assessment, fallopian tube assessment, serum hormone assays and more. These treatments can be invasive and expensive and have undesired side effects or complications (Humaidan et al. 2016). *In vitro* fertilization is the most effective method amongst ART treatments. Mentioned earlier, in 2013 the number of IVF treatments that comprised of women diagnosed with DOR was approximately 61,000. This subset of patients is also one that results in the lowest success in IVF due to lowered number and quality of blastocysts created per IVF treatment and the increased odds of no uterine transfer of embryos due to no blastocyst development or poor blastocyst development. As the percentage of women with DOR, advanced maternal age (AMA) or POF increases each year, there is growing need of the understanding cell culture of gametes and embryos, particularly blastocysts.

IVF is utilized in many different species such as equine for horse breeding, bovine for cattle, mouse for research and human for infertility (Wheeler and Rubessa 2016; Mugnier et al. 2009; An et al. 2017; Feuer et al. 2014). The leading benefit of the treatment is the opportunity of increased pregnancy rates due to the creation of multiple embryos per IVF treatment. Embryos created during such treatments are discarded if development is poor in the trophectoderm or inner cell mass (ICM) cells. The introduction of the mechanical approach this research aims to decrease the number of embryos discarded by utilizing a method that allows for proliferation and adhesion of trophectoderm cells of sibling blastocysts. Ultimately the number of high quality embryos per IVF treatment will be higher, in turn saving embryos that would otherwise be destined for discard. The understanding of response and development when studied in *Mus musculus* provide preliminary evidence and influence the need for study and clinical trials in human IVF.

There are many organisms that have been investigated for embryonic graft transplants including fish and amphibians, where grafts of the embryonic tissue from one embryo were introduced into the developing second embryo of a slightly different and species. For example, grafts of mice embryonic stem cells (ES) into the embryonic stem cells of a rat, which develop into a chimera of both species (Kidder et al. 2008) have been studied for the purposes of disease modeling and the investigation of transgenerational gene expression and epigenetics of gene expression. There is currently no federal funding available for human embryonic stem cell studies, which has limited the transitional studies from lower mammalian species of embryos to develop into human trials. Likely as a consequence, no work has been reported involving rescue of poor quality embryos with graft of TE tissue.

There are baseline diagnostic tests performed routinely by assessing hormone levels in the blood serum. Two of the hormones used to aid in diagnosis of DOR and preliminary ovarian failure are anti-Müllerian hormone and follicle stimulating hormone.

#### 1.3.3.1 Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is a gonadal hormone, member of the TGF-β super family of growth and differentiation factors like inhibins and activins (Koskela and Tapanainen 2016). AMH was initially referred to as Müllerian-inhibiting substance (MIS) and has been known for its role in sexual differentiation since the 1940s (Wilson et al. 1981; Broer et al. 2014; Weenen et al. 2004). The developing fetus will differentiate into a female in the absence of AMH, which allows the Müllerian duct to form into the upper vagina, uterus and oviduct (Hutson et al.1 981; van Rooij et al. 2005; Durlinger et al. 1999). Experiments that were conducted using AMH knock-out in mice brought to light the relationship of AMH and the ovaries whereby AMH inhibits growth of

resting primordial follicles (Broer et al. 2014; Durlinger et al. 2002; Durlinger et al. 1999). Current observations suggest that the absence of AMH leads to a higher rate of recruitment of primordial follicles resulting in exhaustion of the primordial follicle pool (Dewailly et al. 2014).

AMH plasma levels are reflective of the growth of small, non-menstrual cycle dependent primordial follicles (Grynnerup et al. 2012; Weenen et al. 2004; Koskela and Tapanainen 2016). AMH is produced exclusively by the granulosa cells surrounding the immature oocytes in the intra-follicular component producing high follicular fluid concentrations. During early childhood, there is an initial increase in AMH levels, which slowly declines thereafter until it is undetectable roughly 5 years before menopause (Broer et al. 2014). The decline is reflective of the diminishing stock of ovarian reserve. AMH does not accurately detect the quantity of remaining antral follicles or pool of primordial follicles in the ovaries but rather predicts the remaining length of a woman's reproductive lifespan (Weenen et al. 2004; Grynnerup et al. 2012; Safdarian et al. 2012; Visser and Themmen 2014). For the last few years, AMH is thought to be the best endocrine marker for assessing the decline of ovarian reserve in healthy women relative to age (Broer et al. 2014).

Infertility medicine uses AMH as part of diagnostic testing for patients. A strong correlation between AMH and antral follicle count (AFC) retrieved by trans-vaginal ultrasound of the ovaries was observed (de Vet et al. 2002). Furthermore, a strong correlation between AFC, AMH and age, and follicle stimulating hormone (FSH) was described in women undergoing IVF (van Rooij et al. 2005). There have been very few reports on the correlation of AMH and embryonic development, especially the rate of high quality blastocyst formation, for IVF patients. Of late, focus has been on AMH and its ability to predict implantation or pregnancy in IVF treatments versus rate of blastocyst formation. A meta-analysis of such focus had led to a conclusion that there is not a strong association of AMH and implantation potential (Tal et al.

2015). If rate of blastocysts formation can be accurately associated with serum AMH values, then patients with DOR or POF (poor prognosis group) can be counseled appropriately to calculate their rate of success using their own oocytes in IVF.

#### 1.3.3.2 Follicle Stimulating Hormone

Follicle stimulating hormone (FSH) is a gonadotropin, a glycoprotein polypeptide hormone (Vegetti and Alagna 2006; Rinaldi and Selman 2016). The synthesis and secretion of this menstrual cycle-dependent hormone is controlled by the anterior pituitary gland. FSH regulates the development, growth, pubertal maturation and reproductive processes in the body (Smitz et al. 2016). In the antral follicle the granulosa cells, the cells surrounding the oocytes, express FSH receptors. At the onset of the menstrual cycle a small number of antral follicles, usually 2-5mm in diameter are present in the ovaries (Pouwer 2015; Hodgen 1982). This group of follicles continues to grow in response to stimulation by FSH. This process is referred to as follicular recruitment. In the 1970s Brown introduced the concept of the FSH threshold on the basis of serum estrogen levels secreted by the developing follicle growth (van der Meer et al. 1994). According to this concept, increasing FSH concentrations should surpass a distinct level to initiate the final phase of follicular growth that is gonadotropin dependent. It has been hypothesized that follicles demonstrate varying degrees of sensitivity to FSH at the time of recruitment (Scheele and Schoemaker 1996). Therefore, the follicle with the highest sensitivity will benefit the most from increasing FSH levels and gain dominance over the remaining growing cohort of antral follicles in the IVF treatment, leading to maturity and ovulation (Visser and Themmen 2014).

Infertility medicine uses basal FSH serum levels from blood drawn on menstrual cycle days 2-4, to determine ovarian responsiveness to gonadotropin treatment. Unfortunately, some

patients respond poorly to IVF stimulation and are referred to as “low” or “poor responders” (Vega et al. 2016; Oehninger 2011; Cohen et al. 2015). Women in menopause have high FSH levels, above 40 mIU/ml. This occurs because the pituitary continues to produce FSH in the absence of its “inhibitor”, estradiol, normally produced by maturing oocytes. FSH levels in DOR and POF patients will be elevated due to the diminishing reserve of oocytes remaining in their ovaries. The normal FSH value of reproductive women with good fertility potential is usually between 5-9 mIU/ml (Barroso et al. 2001). Increasing values at baseline are suggestive of poor ovarian response to gonadotropin stimulation resulting in a decreased chance of live birth (Namwanje and Brown 2016; Muasher et al. 1988). The focus for FSH in infertility has primarily been on response to stimulation and very limited information is available on basal FSH levels and their predictive value of the rate of high quality blastocyst formation in an IVF treatment. If a correlation is found, this would tremendously aid in counseling IVF patients with accurate success rates using their own oocytes.

### 1.3.4 *In Vitro* Fertilization

Laboratories are currently practicing under the guidelines of the American Society of Reproductive Medicine (ASRM), which is a multidisciplinary organization dedicated to the advancement of the science and practice of reproductive medicine in the United Sates. A set of very strict criteria has led to uniformity across laboratories to maintain high standards in assisted reproductive technology (ART) laboratories. ART has been offered clinically in the US shortly after the birth of the first test tube baby, Louise Brown in 1978 (Steptoe 2015). Since then, there is a continued involvement of regulatory agencies such as the Food and Drug Administration (FDA), Occupational Safety and Health Administration (OSHA), Clinical Laboratory

Improvement Amendments (CLIA), College of American Pathologists (CAP), Joint Commission: Accreditation, Health Care Certification (JACHO), American Board of Bioanalysts (ABB), etc. This regulation had led to a well-established and accepted option of *in vitro* fertilization to the couples or even single persons that desire having a family and are unsuccessful in conceiving. Between years 2004 and 2013, the success rates of IVF treatments have only increased by roughly 7% reflecting single live births by age group ("National ART Summary Report for 2013"). There have been advanced options offered to the ART laboratories such as the newer method of cryopreservation called vitrification versus the rate-cooling method of freezing blastocysts and cleavage stage embryos. Another advancement in IVF is the preimplantation genetic screening or diagnosis of cells biopsied from trophectoderm of blastocysts. This technique involves complete chromosomal screening of the embryos and allows for the selection of euploid embryos for transfer with the promise to increase the chances of pregnancy per IVF treatment up to 80% (Fiorentino et al. 2014; Rubio et al. 2003; Capalbo et al. 2014).

Current methods in the field focus on increasing the success rate per IVF treatment by implementing an exclusion method for embryo selection. PGS excluded the aneuploid embryos from the patient cohort; vitrification excludes the poor quality embryos and offers a preservation solution for the high quality embryos. There has not been a solution proposed that will rescue poorly developing blastocysts from their unfortunate disposition of discard.

### 1.3.5 Embryo Culture

*In vitro* fertilization uses the ability to culture embryos outside of the body in artificially constructed environments. The success of this procedure is reflected by the rate of live birth that is reported by laboratories. For a successful pregnancy to occur, it is essential for a laboratory to

grow high quality preimplantation embryos (Shahbazi et al. 2016; Gardner et al. 2005). Culture media have an impact on the quality of embryos generated in IVF treatments influencing implantation and pregnancy rates (Farquhar et al. 2013; Bungum, et al. 2002). Several manufacturers[2] offer FDA approved IVF culture media commercially available for purchase, each entailing a slightly different composition. Generally, all IVF laboratories maintain logs of quality control (QC) of their selected media, in which embryo culture is performed. This QC can include, but is not limited to:

- pH levels at equilibration with specified $CO_2$ levels (typically 6-7%)
- Temperature at equilibrium (37˚C)
- Rate of mouse embryo blastocyst formation with each lot/batch of media
- Maintenance of a log of lot/batch numbers along with their initial use
- End use and expiration dates

#### 1.3.5.1 Culture Media for IVF

At the infancy of IVF, early 1990s, culture media in the United States consisted of a balanced salt solution, which included glucose and phosphate (Baltz 2012; Wang and Sauer 2006). Subsequently, more complex culture media formulations were introduced with addition of non-essential amino, acids, chelators (EDTA), vitamins and antibiotics (Gardner et al. 1994; Van Blerkom et al. 2014). More recently sequential culture media were formulated after considering the changing metabolism of the embryo from fertilization to cleavage, then from cleavage to blastocyst (Gardner and Lane 1997; Barnett and Bavister 1996; de Los Santos et al. 2015). The cleavage stage embryo is characterized by low levels for biosynthesis (Epstein and Smith 1973),

[2] Manufacturers of IVF culture media in the United States that sell FDA approved media for human use include, but are not limited to: VitroLife, Life Global, Sage and Irvine Scientific.

low respiratory rates (Thompson et al. 1996; Niakan et al. 2012) and a low to limited ability and capacity to utilize glucose as an energy source (Gardner and Leese 1990; Gardner and Wale 2013; Wale and Gardner 2016). In contrast to cleavage stage embryos, post compacted embryos exhibit an increasing rate of biosynthesis and respiratory capacity along with their ability to utilize glucose as their energy source (Wiley et al. 1986; Morbeck et al. 2017).

The selected media for my studies, G-Series-TM media by VitroLife, is a sequential media that is FDA approved for the culture of human gametes and embryos. The first of the two-step media is the G-1 Plus, which is a medium for culture of embryos from the pronuclear stage to the day 3 cleavage stage. This is a bicarbonate buffered medium containing human serum albumin (HSA), hyaluronan and gentamicin as an antibacterial agent. The second of the two-step media is the G-2 Plus, which is a medium for culture of embryos from day 3 of embryonic development to the blastocyst stage. It also contains HSA, hyaluronan and gentamicin. Exact composition of media is found on the certificates of analysis, which are provided for each batch by the manufacturer and demonstrate little to no variability between batches (Appendix B, C).

#### 1.3.5.2 Equilibration pH

It is essential to acquire the correct pH in culture media for gamete and embryo culture during IVF treatments (Steel and Conaghan 2008; Swain 2012; Swain et al. 2016). Culture media are routinely prepared as micro droplets of 40ul per drop in a Petri dish under culture oil (Swain et al. 2012). This design aids in limiting evaporation of the culture media and to maintain its osmolality. The range of pH for culture of human gametes and embryos is 7.2-7.4 (Steel and Conaghan 2008). The pH of the media is controlled by the concentration of carbon dioxide in the

incubation chamber. Carbon dioxide in the incubator is in equilibrium with dissolved carbon dioxide, carbonic acid, bicarbonate and carbonate:

$$CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons H+ + HCO^-_3 \rightleftharpoons 2H+ CO_3^{2-}$$

The media at physiological pH is then approximated by the Henderson-Hasselbach equation (pH =pKa + log10([$HCO^-_3$]/[ $CO_2$]) (Gwatkin 1993; Mioni and Mioni 2015). Relatively large changes in $CO_2$ concentrations (+/- 0.2%) within an incubator cause only minor changes in pH (Gardner et al. 2005). It is very important to validate the media pH for a given media on a regular basis to ensure that it follows the recommendations for the specific media (Michelle Lane et al. 2008). This also ensures that the culture conditions are proper for embryos in any incubator.

#### 1.3.5.3 Partial Pressures of Oxygen and Carbon Dioxide

Embryo development depends on a variety of factors, which include oxygen partial pressure during culture. Oxygen and carbon dioxide partial pressure have a crucial effect on embryo development. Gasses that are utilized by IVF laboratories are either 5-6% carbon dioxide and air (20.9% oxygen) or 5-6% carbon dioxide, 5% oxygen and 90% nitrogen (Waldenström et al. 2009; Nastri et al. 2016). The oxygen partial pressure in the oviduct and uterus for mammalian species has been determined to be in the range of 2-8% (Fischer and Bavister 1993; Archibald 2003). Elevated and non-physiological oxygen concentrations could create unfavorable conditions in the substrate in the form of production of free oxygen radicals causing oxidative stress leading to the occurrence of high rate of fragmentation and arrest of embryos during development (Bedaiwy et al. 2004; Guérin et al. 2001; Peng et al. 2015). In the past there have been controversial results of oxygen stress and its impact on embryonic development. The ratio of oocytes collected per case compared to embryos viable on day 3 of culture has not shown to be significantly different

under ambient or low oxygen (5-6%) (Dumoulin et al. 1999; Peng et al. 2015; Swain 2015). However, the rate of blastocyst formation was negatively affected with lower percentage of embryos developing to blastocysts when culture was maintained in ambient oxygen concentration (Kelley and Gardner 2016; Dumoulin et al. 1999; Yang et al. 2016) . Not only were there improvements in blastocyst formation, but also improved pregnancy rates, implantation rates and birth rates were found from treatments whose culture was performed at low oxygen concentrations. It is well understood and accepted that the overall outcome of IVF treatments is improved with low oxygen stress during culture. However the narrow window of *low stress* has yet to be delineated.

### 1.3.6 Association/Dissociation of Sibling Trophoblast Cells

Cell adhesion is maintained primarily by transmembrane proteins called cadherins (Mui et al. 2016; Simopoulou et al. 2014). Cadherin class of proteins comprise of hundreds of cadherins, however, E(epithelial)-cadherin, is the first of its class to be expressed in the mammalian embryo (Watson and Barcroft 2001; Zaidel-Bar 2013; Gilbert 2014). These proteins also play an important role in cell forming adherens junctions and bind cells together. Synthesized as polypeptides, cadherins undergo many post-translational modifications to ultimately form the proteins, in which cell to cell adhesion and recognition is functional (Harris and Tepass 2010; Mui et. al 2016). These polypeptides are approximately 750 amino acids long with a small transmembrane component and a bulk of the protein is extracellular. Cadherins are calcium ($Ca^{2+}$) ion dependent in their functionality (Yap et al. 2015). Cell-to-cell adhesion is mediated by the extracellular component or domain of the protein, whereas the intracellular cytoplasmic tail integrates with other signaling and adaptor proteins referred to as cadherin adhesome (Zaidel-Bar 2013). This protein plays a

critical role in morphogenesis and homeostasis and has 3 major functions in animals (Basilicata et al. 2016; Zartman et al. 2009; Priya and Yap 2015). First, cadherins provides adhesion tension, which lowers interfacial tension at the cell to cell contact. Second, they signal to the actomyosin cytoskeleton to reduce cortex tension, which reduces interfacial tension at contact. Third, cadherins stabilizes the contact by resisting mechanical forces that work against this contact by force. Cadherins are involved in determining cell shape and position of different species. For example, in zebra fish and *Caenorhabditis elegans*, cadherins are implicated in germ cell positioning (Babb et al. 2001; Callaci et al. 2015). In Drosophila they are involved in the oocyte positioning within the oocyte chamber and in *Drosophila* and *C. elegans*, they are involved in the epithelial folding and mesoderm/endoderm cell internalization (Maître and Heisenberg 2013; Zartman et al. 2009).

For the purposes of cell culture, media are kept close to physiological conditions. Removing calcium and magnesium ions from cells or tissue would weaken the cadherins in cell bonds and cause cell-to-cell dissociation (Ohnuma et al. 2014; Heng et al. 2009). This exact technique is the underlying mechanism for the out-dated method of embryo biopsies in human IVF. In the past, embryos were biopsied on IVF treatment day 3 at the cleavage stage of development. The embryo was then placed in a calcium and magnesium-free solution and allowed to equilibrate for 10-15 minutes. A hole was then created in the zona pellucida of the embryo and a single cell was pulled away using micro-tools under an inverted microscope (Olympus IX70) (figure 1.5). The weakening of the cells, in the absence of calcium and magnesium allowed the technician to remove this “biopsy” or cell without causing damage to the remaining embryo. Once the biopsy was removed, the embryo would be placed back in the normal culture media and the cell bonds would re-associate (Łukaszuk et al. 2015; Heng et al. 2009; Kawakami et al. 2006). The embryo would continue its cell divisions as normal. This technique is no longer used due to the

limitations of biopsy[3] and removal of only one blastomere versus 5-6 trophoblast cells biopsied at the blastocyst stage. This is due to the availability of more cells at a later stage leading to a more robust preimplantation genetic screening analysis (discussed in Chapter 4). Many of the commercial cell media companies offer FDA approved calcium and magnesium free solutions for this specific purpose. This technique has not been applied to the current TE biopsy of the day 5 embryo due to the availability of the laser[4] lens, which emits pulses for ablation of the cells to perform a biopsy.

### 1.3.7 Examples of Chimeric Growth

The basis of the proposed sibling trophoblast study is the theory that the trophectoderm cells are differentiated and are programmed to support the ICM regardless of mosaic or chimeric state. Many experiments using mapping techniques have been performed to determine the ultimate fate of a single cell at the embryonic stage to the tissues and organs that arise from it at an adult stage. Most model organisms have been mapped – e.g. the zebra fish and *C.elegans*. Viable chimeras produced from mouse embryos have been reported (Solter 2010; Hizanidis et al. 2016). In this case, cleavage embryos were removed from their zona pellucida and placed together in continued culture. The resulting embryos were an integration of the two cell lines, chimeric. A chimera is an organism with cells from two genetically different zygotes. These embryos were transplanted to the uterus of the foster mother mouse and live births were reported. The adult chimeras were then mated with normal mice and were proven to have uncompromised fertility. No adverse events were reported in development.

[3] Day 3 blastomere biopsy and its limitations are beyond the scope of this project. (For review, see Magli et al. 2016)

[4]. The Saturn Laser Lens is used for all biopsy procedures in the proposed studies. 1480nm / 400mW solid state diode laser. Pulse length range 0.005-2.0ms / 5-2000µs. http://www.research-instruments.com/saturn-5/

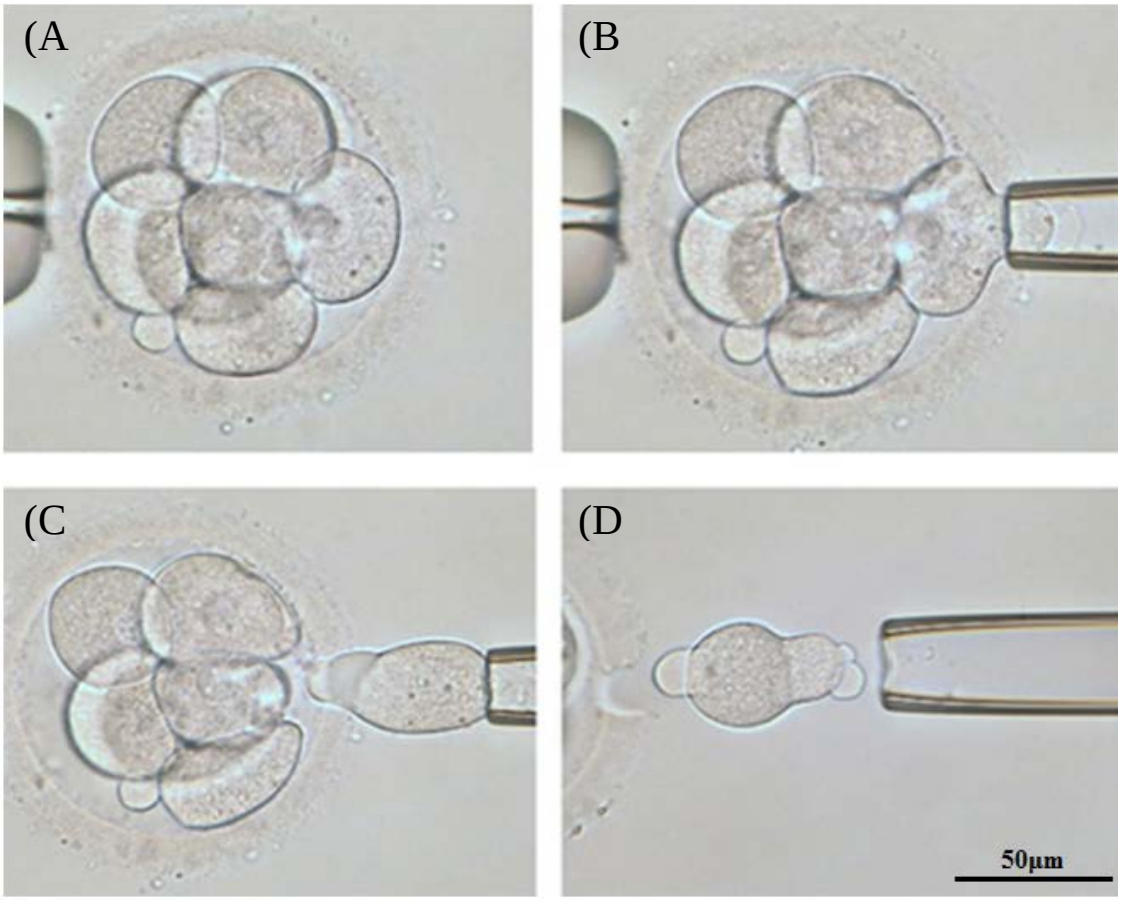



Figure 1.5. Day 3 embryo biopsy. On day 3 of embryo development, an embryo is between 6 and 8 cells (A). The zona pellucida is ablated and a single cell is removed from the embryo using micromanipulators (B, C). This single cell serves as a "biopsy" and is then sent for preimplantation genetic screening (D). Courtesy: Dallas IVF

Attempts have been made to knock out a gene of interest in a mice strain and constructed chimeras (Cheatham et al. 2009). The chimeras were produced by fusing the embryonic cells of the two mice strains to construct a mouse with the desired knock out (Cheatham et al. 2009) . Are chimera's possible in humans? There is even evidence that human embryos can form chimeras (Benirschke et al. 1972; Ali et al. 2017; Bens et al. 2017). Some individuals have two genetically different cell types (XX and XY) with in the same body. Each with its own set of genetically defined characteristics. Usually individuals that have this complete fusion of embryonic cells that express into fetal development are developed into hermaphrodites that have sex organs of both

male and female. Then there are other cases such as monozygotic twinning, particularly cases that develops into one chorion (separating fetuses) and one placenta (shared between the two fetuses). Pregnancies that comprise of twins sharing the same placenta have concerns that are similar to the ones in this research such as same blood supply shared between two fetuses. There are reported live births of twin gestations that have lived healthy lives, have full reproductive potential and have not been developmentally impacted (Bui et al. 2015; Gielen et al. 2006).

In an effort to reduce the risks associated with transferring a chimeric or abnormal embryo, IVF laboratories in the country offer pre-implantation genetic screening of embryos created in IVF treatments. The ability to test the genetic makeup of an embryo reduces the likelihood of an abnormal pregnancy, miscarriage or no pregnancy. This testing is now discussed.

### 1.3.8 Preimplantation Genetic Screening

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) have been used in IVF to detect unbalanced chromosome copy numbers or single gene disorders in embryos (Zhu et al. 2014; Capalbo et al. 2014; Sermon et al. 2016; Staessen et al. 2008). There are three widely used methods of testing offered to patients undergoing IVF, which include array comparative genomic hybridization (aCGH), small nucleotide polymorphism (SNP) array, and next generation sequencing (NGS). Chapter 4 study focuses on the differences in the pregnancy rates achieved when embryos were transferred using the various methods of screening or diagnosis.

#### 1.3.8.1 Array Comparative Genomic Hybridization

Often referred to as a microarray or array CGH, this test can detect small changes in the

chromosomes such as micro-deletions or duplications (Valli et al. 2015; Harper and Harton 2010; Kotdawala et al. 2016). The micro-chip based array exploits the ability of the DNA molecule to bind specifically to another DNA molecule. The microarray comprises of tens of thousands of short sequences of DNA called probes that are arranged in a precise grid on a glass slide referred to as a "chip". The biopsy cell samples from the embryos are lysed to retrieve the DNA, which is amplified and then digested into short fragments (Rubio et al. 2013). These fragments are then labeled with a colored fluorescent dye. The reference DNA or control, with no genetic anomalies is then labeled with a different color fluorescent dye. The test and control samples are mixed together and hybridized to the probes on the microarray chip. A scanner then measures the amount of the two different dyes on each probe. Ratios of the dyes are calculated by analytical software to determine if the test sample has the correct copy number of chromosomes. Duplications and deletions of whole chromosomes or large portions of chromosomes can be detected by this method (Adler et al. 2014; Kotdawala et al. 2016; Fragouli and Wells 2012). The limitations of this method are that aCGH cannot detect uniparental disomy, mosaic cell lines, small segmental deletions or duplication or trisomy across all chromosomes (Liu et al. 2011; Handyside et al. 2012; Handyside 2013).

#### 1.3.8.2 Small Nucleotide Polymorphism Microarray

Small nucleotide polymorphisms are small areas of the genome within, which a single nucleotide in a sequence varies within a population. SNP arrays detect uniparental disomy by comparing the variable regions of DNA if parental blood or buccal samples are provided (Ahmad and Iqbal 2012). A typical array will have hundreds of thousands of SNPs to access using validated software that can determine how many of each chromosome number was inherited by the embryo

from each gamete donor (Schroeder et al. 2013; Zhu et al. 2014; Adams et al. 2012). This is performed by analyzing both quantitative data and analyzing the expected combination of inherited chromosomes and comparing them with the actual outcome (Harper and Harton 2010). After whole genome amplification is performed on embryo biopsied cells, the DNA is labeled with red and green fluorescent molecules with one version of the SNP in red and the other in green (Dahdouh et al. 2015). The DNA is then assessed for intensity of the fluorescent signals as well as the SNP calls. This is compared with the control samples (DNA of the gamete donors) by a validated software, which establishes diagnosis of disease and confirmation of inheritance of chromosomes and copy chromosomal number (Harper et al. 2010). SNP arrays offer options that are not available with aCGH. For example, in addition to detection of uniparental disomy, SNP arrays can perform simultaneous testing of single gene disorders along with complete chromosomal screening (Singh et al. 2015; Li et al. 2014). Due to these added benefits, this method of screening and diagnosis has been utilized more often for IVF (Fragouli and Wells 2012).

#### 1.3.8.3 Next Generation Sequencing

Next generation sequencing (NGS) is a new emerging technology that is yet to be validated for clinical use (Muzzey et al. 2015; Visser et al. 2017). It is a high throughput based non-Sanger sequencing technology that allows sequencing of increased number of samples simultaneously (Meller 2015). NGS offers enhanced detection of partial or segmental aneuploidies, detection of mosaicism in multicellular samples and the potential of automation of sequencing library to reduce hands on time and human error (Fiorentino et al. 2014; Marshall et al. 2017).

## 1.4 Raising the Bar for IVF Treatments

The Society of Assisted Reproductive Technology and the CDC reports diminished ovarian reserve as the second leading diagnosis for IVF accounting for 31% of ~232,000 IVF treatments in the United States in 2015 ("2015 ART National Summary Report" 2017). This diagnosis is accompanied by challenges of poor prognosis, as previously discussed. IVF treatments are emotionally and financially costly and can lead to added stress for patients presenting with DOR. It is therefore, my aim to understand and define culture conditions, stimulation treatment effects and embryo selection methods, which can optimize success for patients with diminished ovarian reserve and broaden the scope of the field by providing an experimental inclusion method for poorly developed blastocysts, which may increase rate of high quality blastocysts development leading to overall increase in treatment success.

The next chapters outline and investigate methods for optimization of IVF. Chapter 2 discusses the statistical methodologies applied to chapters 3-6 with concentration on the power of analysis with interactions. Chapter 3 explores embryonic adaptability in the form of integration of sibling graft cells in the mouse to aid in blastocyst growth built on the foundation of chimeric organismal development. Chapter 4 focuses on the more suitable and constructive approach to pre-implantation genetic screening methodologies and limitations associated with each technique for the most favorable application for infertile patients. Chapter 5 concentrates on the power of diagnostic factors such as basal follicle stimulating hormone and anti-müllerian hormone combined with actionable variables during an IVF treatment cycle such as gonadotropin dose for ovarian stimulation and day-of-trigger to build a prediction model for use with IVF treatments improving blastocyst development. Chapter 6 investigates the fundamental, yet paramount principals of medium pH, $pCO_2$ and $pO_2$ to discern association of subtle batch-to-batch variability

and their implications on developing embryos. Thus, you are presented with some of the most leading factors associated with human IVF focusing on blastocyst development, especially for DOR and POF patients. Chapter 7 integrates findings to show how individualized and tailored approach to IVF can maximize blastocysts developmental success and lessen the financial and emotional burdens for infertile patients.

# CHAPTER 2

# CRITIQUE AND EXPLANATION OF STATISTICAL METHODS

## 2.1 Introduction

Conventional methods to analyze scientific experimental data have been successfully and effectively used for many years. Examples of these methods include analysis of variance (ANOVA) and Chi-square tests. These methods are a part of statistical modeling, which formalizes relationships between variables in the form of mathematical equations (Khuri 1999; Rutherford 2011). ANOVAs (one, two and three-way) are strong analyses that can identify variability in observations. They are effective for a variety of data-sets that have 1-3 variables, providing significance values to indicate importance. While this conventional method provides a strong power of analysis, its application and effectiveness in large data sets comprising bioinformatics is limited.

### 2.1.1 ANOVA Analysis

Often, researches apply one-way ANOVA for a given factor (primary) for each level of the other factor (secondary), repeatedly. This strategy suggests that their interest lies in the secondary factor, therefore implying that one-way ANOVA is the appropriate methodology. As an example, if patient age was the primary factor and pregnancy was the outcome, one-way ANOVA would likely find a significant relationship. However, embedded variation within age such as type of infertility (i.e., diminished ovarian reserve, endometriosis, hypogonadism, hypothyroidism, male-factor, etc.) would be unaccounted for. The multiple interactions of diagnosis on patient age are not detectable by the one-way ANOVA due to its limitations in identifying weights of interactions between explanatory variables on final outcome.

Taking critiques of statistical methodology a step further, application of two-way ANOVA analysis requires the experimental data to have the same number of combinations of observations among the variables of interest and the outcome variable. If this combination is out of balance, the analysis is then conducted without interactions. This model provides main effects (no-interactions) to determine effect of one factor from that of another. The prior, interaction model, includes the relationship between variables and thus accounts for interactions (Kim 2014). As an example, if the diagnosis of diminished ovarian reserve was the primary factor with number of fertilized oocytes in treatment as the secondary factor and outcome is observed as pregnancy, the primary factor consists of variation within the population, i.e. age of patient. The lack of accountability of interaction analysis of variables within a study can easily result in unexpected and surprising results. In a case like this, researches often choose to lump patients into age categories like 20-34 years, 35-37 years, 38-40 years, etc. as is reported by SART for *in vitro* fertilization in the United States (see Chapter 1). Once grouped, this data set can be analyzed in groups, including a couple of variables in each analysis and output is often compared across the age categories. As a consequence, lumping of maternal age within groups to create categories is misleading because it determines the slope of the curve within a narrow window of age, expressing the data as a linear model. If maternal age was analyzed as a continuous variable compared to categorical lumping, the slope is drastically different for pregnancy outcome resulting in a non-linear and parabolic curve, consequently changing interpretation of the final analysis.

When more than three variables are involved per observation, which is emblematic of bioinformatics study data, the analysis becomes overwhelmingly complex. Some scientists may split the data in parts to perform multiple ANOVAs as their choice method of analysis. For example, a dataset, which has six variables (A-F) may be analyzed in 3 runs: variables A and B

against outcome, then variables C and D against outcome, and finally variables E and F against outcome. A post-hoc multiple comparisons may be implemented as if the three independent analyses were performed as one set. Selecting certain variables by choice per treatment to include in an ANOVA analysis in groups compared to all variables against outcome discriminated in one analysis results in a loss of interactions among variables. This masks critical information on the weighted influence of variables on outcome or each other. The results are consequently mis-interpreted giving significance to a variable that is possibly only significant because it was coupled with a highly insignificant variable in the analysis which otherwise, is truly insignificant in the presence of another variable with greater weight on outcome. Alternately, significance due to combined effect of variables can be missed if the grouping has not included interacting variables.

Specifically in my dataset for Chapter 6, a total of 16 variables were included per observation. As is discovered by the reader, the focus of the study was to understand outcome in the form of blastocyst development rate related to culture conditions to include pH, partial pressures of carbon dioxide and oxygen. By graphically representing the output readings of $pCO_2$ against pH by medium type the inverse relationship of carbon dioxide and pH is depicted (figure 2.1). Outliers in the data set are plotted in this figure and the majority of the data readings were consistent as represented by batch number. The top panel shows 2 batches plotted for primary culture medium G1$^{TM}$ Plus (VitroLife) (figure 2.1A) and lower panel shows 3 batches for secondary culture medium G2$^{TM}$ Plus (VitroLife) (figure 2.1B).

Of critical importance in justifying the analyses used in this thesis, performing ANOVA on these two variables may or may not show significance between batch and readings. However, the results of this analysis are invalid since the interactions between incubator number, incubator temperature, age of female producing oocytes, infertility factor associated with the patient's

oocytes and some of the other variables directly influencing final outcome have not been included. As documentetd in Chapter 6, an effect on outcome was found, however, it was not due to batch-to-batch variation, as I conclude by evaluating figure 2.1. Instead, I found pCO2 and $pO_2$ to have a stage dependent and patient diagnosis dependent effect on final outcome. ANOVA would be unsuccessful in identification of this critical finding.

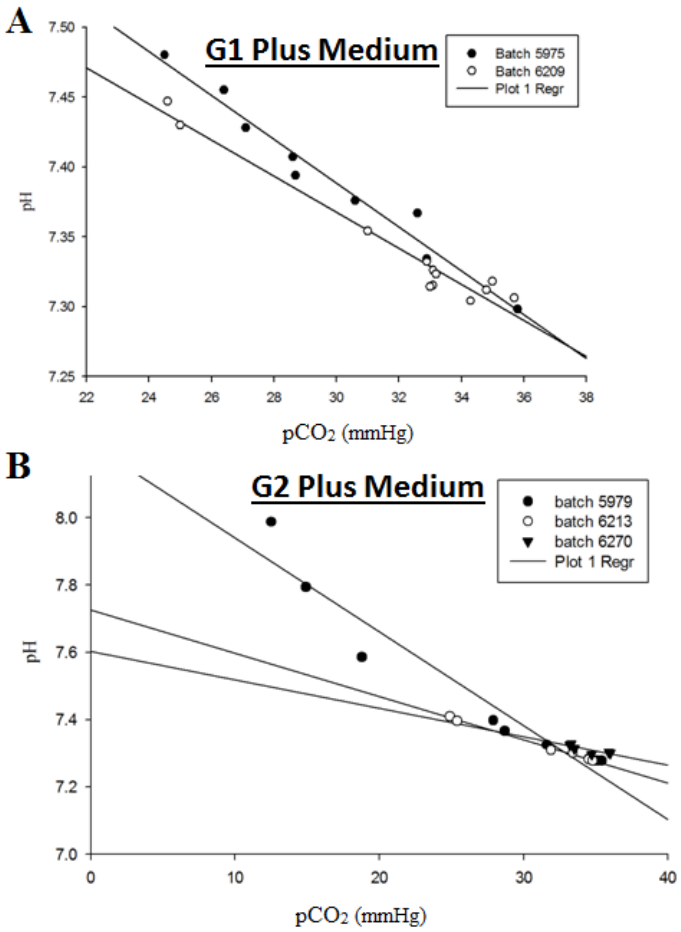



Figure 2.1. Comparison of pH and $pCO_2$ by medium. G1 Plus is the primary medium supporting embryonic development to cleavage stage, where-as G2 Plus supports embryonic development from cleavage stage to blastocyst stage. A plot of two batches for G1 Plus and three batches of G2 Plus demonstrates the inverse relationship of pH and $pCO_2$ and regression lines for observations are plotted. Regression lines are plotted and the slopes of the lines are influenced by outliers. This subtle difference can alter the slope of the curve and identify this as significant. The differences in batch characteristics, when examined without other variables in the study, incorrectly analyze significance due to missing interactions. This form of analysis for a large bioinformatics datasets results in false positive findings.

### 2.1.2 Chi-Square Analysis and Fisher's Exact Test

Analysis of raw data in chapter 4 (Pre-implantation genetic screening study) for comparison of pregnancy and implantation outcomes to reference group was made through the Pearson's chi-square and Fisher's exact test (Ugoni and Walker 1995), initially. Results of $P \leq 0.05$ were considered significant. Pearson's chi-square analysis (Khuri 1999; Pandis 2016) determined that the clinical pregnancy rates of the reference and treatment groups in the outcome for single embryo transfer (SET), double embryo transfer (DET) and overall clinical pregnancy rate categories were not statistically different (table 2.1). Individuals in all treatment groups had a mean female age that was not different (P=0.073) (determined using one-way ANOVA) (Kim 2014). The mean female age in the reference group was significantly different than the treatment groups (P<0.001) (determined using ANOVA), consisting of a younger patient population (group A was 32.4 ±3.7 years, B was 35.5 ± 4.1 years, group C was 35.1 ± 4.4 years and group D was 36.4 ± 3.5 years).

Table 2.1. Comparison of pregnancy and implantation outcomes to reference group

| | Group A | Group B | | Group C | | Group D | |
|---|---|---|---|---|---|---|---|
| Parameter | No PGS | aCGH | *P* value | NGS | *P* value | SNP array | *P* value |
| # of treatments with SET | 142 | 41 | -- | 37 | -- | 49 | -- |
| # of treatments with DET | 81 | 15 | -- | 13 | -- | 24 | -- |
| Clinical pregnancy rate with SET % (n) | 59.8% (85) | 68.3% (28) | 0.448[a] | 51.4% (19) | 0.455[a] | 67.3% (33) | 0.448[a] |
| Clinical pregnancy rate with DET % (n) | 66.7% (54) | 86.7% (13) | 0.219[b] | 53.8% (7) | 0.369[b] | 70.8% (17) | 0.893[a] |
| Overall clinical pregnancy rate % (n) | 62.3% (139) | 73.2% (41) | 0.172[a] | 52.0% (26) | 0.234[a] | 68.5% (50) | 0.418[a] |
| Overall implantation rate % (n) | 49.5% (151) | 63.4% (45) | 0.051[a] | 50.8% (32) | 0.981[a] | 56.7% (55) | 0.276[a] |
| Overall ongoing pregnancy rate % (n) | 55.6 % (124) | 66.1% (37) | 0.205[a] | 46.0% (23) | 0.205[a] | 60.3% (44) | 0.060[a] |
| Overall miscarriage rate % (n) | 10.7% (15) | 9.8% (4) | 0.938[a] | 11.5% (3) | 0.827[a] | 12% (6) | 1.000[a] |

*SET* single embryo transfer, *DET* double embryo transfer
[a]*P values* determined using Chi-square analysis.
[b]*P values* determined using Fisher's Exact test.

The same parameters outlined in table 2.1 were re-analyzed for significance using the Pearson's chi-square test to compare the treatments to each other. Surprisingly, no significance occurred in the clinical pregnancy rate across all three categories (table 2.2). Although the overall

chemical pregnancy and implantation rate were the highest in group B (aCGH) per embryo transferred (figure 2.1) there was no significant difference in outcomes across treatments in this form of analysis. Due to the unexpected lack of significant differences in outcomes between treatments, the data were re-evaluated using a more powerful analytical method factoring in the interactions of the variables (Chapter 4). The random forest analysis was used in this chapter and interactions that weighed heavily on outcome were identified with the highest weighted variable being female age. This interaction and others were missed using Chi-square and fisher's exact analysis on this type of data. The results were incorrect and significance between treatments and their dependence of female age was not identified until the application of random forest.

Table 2.2. Comparison of pregnancy and implantation outcomes amongst screening groups

| Parameter | Group B aCGH | Group C NGS | Group D SNP array | *P* value |
|---|---|---|---|---|
| # of treatments with SET | 41 | 37 | 49 | -- |
| # of treatments with DET | 15 | 13 | 24 | -- |
| Clinical pregnancy rate with SET % (n) | 68.3% (28) | 51.4% (19) | 67.3% (33) | 0.229 [a] |
| Clinical pregnancy rate with DET % (n) | 86.7% (13) | 53.8% (7) | 70.8% (17) | 0.161 [a] |
| Overall clinical pregnancy rate % (n) | 73.2% (41) | 52.0% (26) | 68.5% (50) | 0.055 [a] |
| Overall implantation rate % (n) | 63.4% (45) | 50.8% (32) | 56.7% (55) | 0.337 [a] |
| Overall ongoing pregnancy rate % (n) | 66.1% (37) | 46.0% (23) | 60.3% (44) | 0.878 [a] |
| Overall miscarriage rate % (n) | 9.8% (4) | 11.5% (3) | 12% (6) | 0.926 [a] |

*SET* single embryo transfer, *DET* double embryo transfer
[a]*P values* determined using Chi-square analysis.

Against this backdrop, I explored the even stronger power of analysis that is offered by machine learning, namely so-called "predictive modeling". Machine learning roots from computer science and artificial intelligence with systems that learn from experimental data compared to analysis based explicitly on programmed instructions (Baştanlar and Ozuysal 2014; Deo 2015). A relatively new field, machine learning provides an impressive processing power of computers programmed to make successful predictions given the complexity of the data. For example, with the World Wide Web at our fingertips, the public uses search engines like Google to perform

queries on topics of interest. Sites such as this provide tools to filter results, which can be billions of web pages. This filtration tool is used to sift through the results to find patterns most relevant to the query and provide a condensed list of results that are most applicable.

This type of process has evolved through fields like computer science and physics and are often referred to as “machine learning”, “artificial intelligence”, “data mining”, “predictive analysis”, etc. (Kuhn and Johnson 2013). This form of artificial intelligence is programmed to “learn” as the characteristics of the data set change. For example, we rely heavily on our email filters to accurately identify certain types of emails as spam. As the user selects emails in the inbox as spam, the algorithm learns from patterns within emails that characterize them as spam for the user. Ultimately, the user is satisfied with the filtering capability of the software and checks the spam folder less frequently.

Understanding the power of analysis that predictive modeling provides for datasets involving 10 or more variables I chose Random Forest and Evtree analysis methods for my studies. I now discuss the methodologies involving each of the analyses.

## 2.2 Statistical Methods Employed in This Thesis

### 2.2.1 Random Forest Analysis

The Random Forest algorithm was created by Breiman (2001). The basic unit and foundation of this and the Evtree (discussed next) is a “tree” with one main “node” or the trunk from which all possible combinations stem. This tree then splits into smaller branches with the split at each branch referred to as a “node” and the “leaves” of each branch represent a homogeneous sub-group (figure 2.2). This tree-based regression approach creates thousands of trees with datasets. Each is unique, slightly different from the others created in the analysis.

Random forests randomly selects predictions at each split starting with the most weighted variable at the top creating splits called “nodes”. Each node then produces two subgroups called “leaves”. The algorithm learns and tunes using the similarities prevalent in each tree generated and produces a final tree with split rules. The split rules are created by maximally discriminating between the two subgroups (leaves) providing the split (node), however maintaining the most homogeneity within each of the independent subgroups. The variables are weighted for importance using the mean decrease Gini index, a coefficient to measure the contribution of each variables to the homogeneity of the nodes and leaves in the final random forest model (Menze et al. 2009; Handyside et al. 2012). For each split the Gini index coefficient of the resulting node is calculated and a comparison is made to the original node ultimately producing a weight of each variable in the final decision tree or predictive model.

The predictive model uses multinomial logistic regression, which can predict the categorical membership of a dependent variable based on multiple independent variables (Starkweather and Moske 2011). This method that utilizes boot strap re-sampling, by which the algorithm includes 70% of observations in the dataset to compose the final tree labeled as “in bag” and the remaining 30% as “out of bag” observations are fitted through the final tree for accuracy of the prediction. This approach is used to compose the final tree minimizing over fitting the model (Breiman 2001; Gromski et al. 2014). The random forest algorithm perturbs sample through re-sampling, dropping the variable of lowest importance. All interactions that influence outcome are accounted for within the iterations conducted by the analysis. The algorithm has a tuning parameter call the $m_{try}$, which is recommend as one-third of the number of predictors (Breiman 2001). Since the analysis is intensive, the number of randomly selected predictors $k$, should also be considered. The algorithm tunes $m_{try}$ by the value of $k$ to maximally produce optimal results that do not over

fit the data. The mathematical interpretation of the analysis is explained by Breiman (2001), naming random forests as more efficient than other bagging tree analysis methods since it requires fewer variables to predict each split in the tree building process. This method, on the other hand requires more trees, however, the combination of parallel processing gives the random forests higher efficiency computationally than other methods involving boosting (Kuhn and Johnson 2013).

Although random forests provide a strong and robust analysis, other approaches provide alternate, and powerful, computations which can be conducted in a shorter amount of time. I now discuss one such method known as the Evtree.

### 2.2.2 Evtree Analysis

Evtree, short for evolutionary tree, analysis is an alternative method to compute predictive modeling based on a genetic and global optimization method (Grubinger et al., 2014). Inspired by Darwinian evolution, the algorithm employs concepts such as inheritance, natural selection and mutation to calculate a decision tree. Population based trees are simultaneously fitted by *variation operators* called *mutation* and *crossover,* which merges various solutions. Ultimately, the "survivor" selection is an evolutionary process, in which the quality of the population is increased over time (Eiben and Smith 2007). This analysis uses Bayesian Information Criterion (BIC) to fit the survivor model (Volinsky and Raftery 2000). Initialization of the analysis is conducted by a valid, randomly generated root node. From this point, iterations are only selected once to be altered by the variation operators to give rise to the parent nodes. The evaluation function of the algorithm formulates, through statistical and mathematical computations, the requirements for the population adaption. In this approach each parent solution competes for its place in the population with the

offspring. The split rules are determined for the offspring population to be discriminate from the parent population, however mutated from the sibling population in terms of nodes on the tree. Finally, termination of the analysis is reached when the top 5% of the generated trees are stabilized for a minimum of 100 iterations.

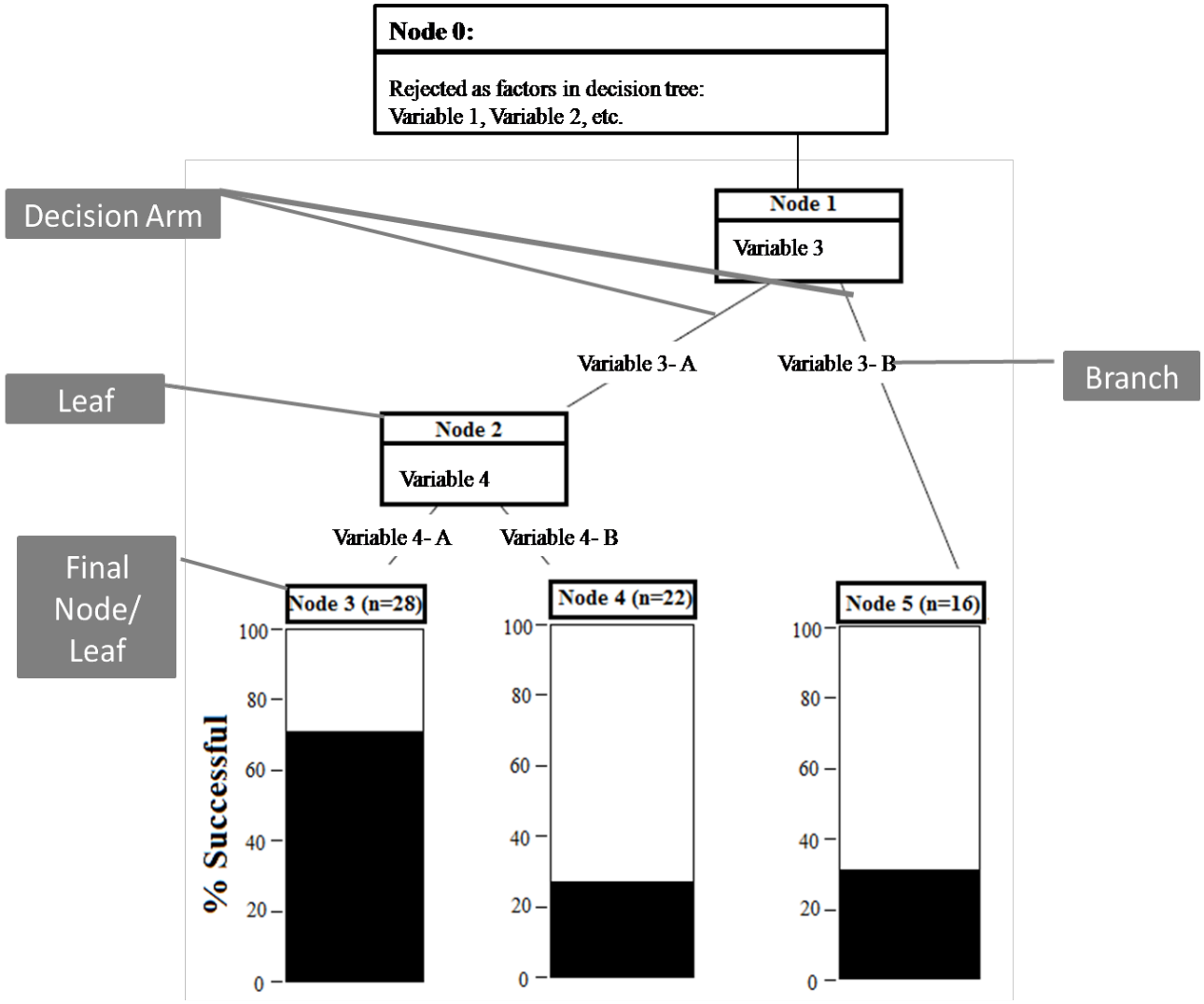



Figure 2.2. Decision tree. This figure is representative of a predictive modeling decision tree. The gray boxes indicate the terminology associated with the various parts of the model.

Evtree does not perturb variables due the evolutionary nature of the analysis. It does not use bootstrap re-sampling or cross validation like random forests. Instead the Evtree package in R

software, implements an evolutionary based algorithm used to learn the classification of global optimality of a dataset.

### 2.2.3 Receiver Operating Characteristic Curve

Receiver operating characteristic curves are a tool used for the evaluation of classifiers in applications utilizing datasets for bioinformatics (Robin et al. 2011; Hajian-Tilaki 2013). This curve is commonly used in predictive modeling analyses, such as random forest and Evtree, for supplemental verification of the accuracy of the prediction model. Commonly referred to as the ROC curve, this graphical plot illustrates the strength of the diagnostic ability of a binary classification (positive/negative) system as the threshold of this system's discrimination varies (Powers 2011) . The curve is created by plotting the true positive rate against the false positive rate of a model over varying thresholds. The true positive rate is known as sensitivity or the probability of detection in machine learning (Froud and Abel 2014). Similarly, the false positive rate is known as specificity and can be calculated (1-specificity). In a prediction model using the probability distributions of detection rate and specificity, the ROC curve is generated by plotting the area under the curve as the probability of distribution up to the discrimination threshold of the detection probability on the x-axis and the distribution function of sensitivity on the y-axis.

The ROC curve maps output in classes. In a binary classification system, there are two outcomes: positive and negative. If the prediction model predicts an actual positive value as positive it is plotted in the top left quadrant. However, actual negative observations which are predicted as positive by the model are plotted in the top right quadrant and are false positive also known as type I errors. Actual positive values predicted as negative are plotted in the bottom left quadrant, a type II error. Finally, the bottom right quadrant represents actual negative values

predicted to be negative by the model. Figure 2.3 is an overlap of the binary classification table with a ROC curve. The “area under the curve” (AUC) is referred to as the trapezoidal area under the curve to the 45˚ diagonal line stretching from the bottom left (100% specificity, 0% sensitivity) to the top right (0% specificity, 100% sensitivity). The further in the top left quadrant the ROC curve stretches, the stronger the predictability power of the model resulting in higher chances of the model’s accuracy when it predicts an actual positive condition as positive (Kuhn and Johnson 2013). An AUC of 1 represents a perfect test with 100% predictive accuracy, while an AUC of 0.5 represents a poor test, in which the prediction accuracy is no better than chance. AUC of 0.6 represents a model that predicts 10% greater success than chance and is the lowest measure of an acceptable prediction model.

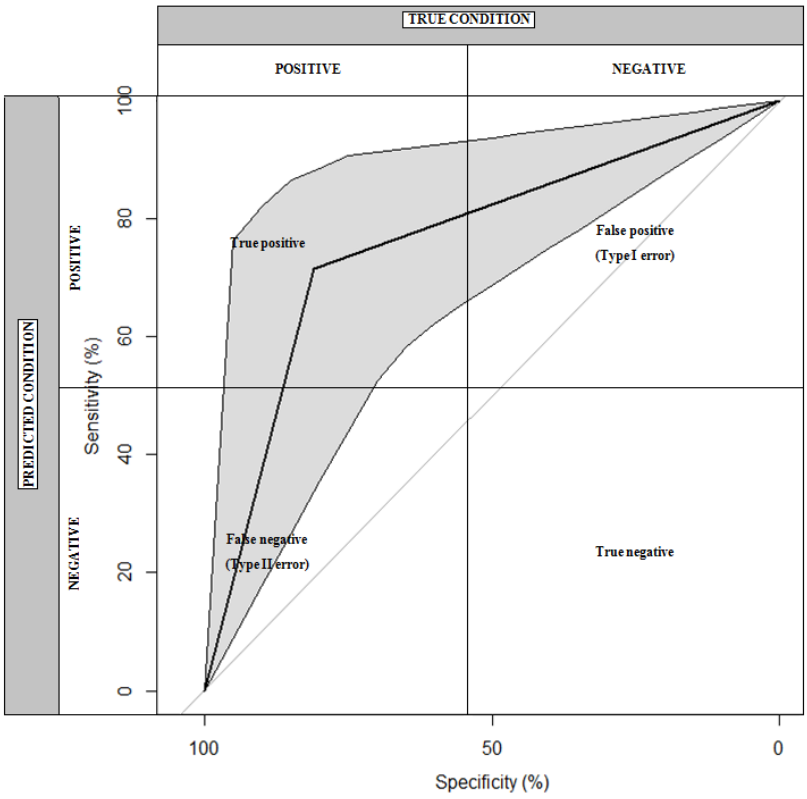



Figure 2.3. Receiver operating characteristic (ROC) curve. A sample curve is superimposed on a prediction table. The 45˚ line demonstrates the role of chance for any predicted condition to result in the true condition. The gray areas of the curve represent confidence intervals where the lowest predictive success and the highest predictive success of the model can be calculated.

The ROC curve is an analysis performed on the accuracy of the entire and final prediction model, representing the accuracy of any final node/leaf on the tree. ROC curves are calculated to consider all factors acting simultaneously and instantaneously on outcome as the discrimination threshold varies of the final prediction model. Therefore, ROC curves are a useful tool in understanding the accuracy of prediction models.

### 2.2.4 Confidence Intervals-95%: Measure of Effect Size

Confidence intervals (CI) are an important measure of certainty and they determine the accuracy of the effect size of treatment to determine the model's practical significance (Banjanovic and Osborne 2016; Davison and Hinkley 1997). Effect size of a treatment in a predictive model will provide the magnitude of the power of a finding and the CI provides the reliability potential of a decision in the predictive model. I used 95% confidence intervals for all analysis of prediction models in my studies using a function called 'boot.ci' in R software, where CI were calculated. This function produces statistics with re-sampling (Canty and Ripley 2017). Larger re-sampling will ensure higher accuracy of the CI. For example, figure 2.3 is a computer code that was prepared for analysis performed in chapter 4 (PGS comparison study) where one of the goals was to determine if treatment with PGS (Group B, C, or D) was more beneficial than no treatment (group A, reference). The re-sampling size was set to 5000 iterations. The resulting 95% CI was reported as: 0.6358 - 0.7706. A lower limit of CI at 0.5000 would indicate that the results are no better than chance, (50%) accuracy. In this example, the lower limit of CI (0.6358) indicates that the decision of the predictive model is 13% greater success than chance alone at its lowest accuracy. The higher limit of CI (0.7706) indicates that the decision of the predictive model is 27% greater success than chance alone at its highest accuracy.

Confidence intervals (CI), being an analysis on effect size, generally becomes narrower as the sample size increases. Data with smaller sample sizes usually will have large variability in results compared to larger sample size data. CI will result in a true value of the data correlation when sample sizes are larger. A narrower range of CI will indicate a higher reproducibility compared to a wider range.

```
Group A (x0) compared to
  Group B (x1):
call: roc.default(response
  = response, predictor =
  predictor, levels =
  c("x0",      "x1"), plot =
  T)
Data: predictor in 223
  controls (response x0) <
  56 cases (response x1)
Area under the curve:
  0.7041
95% CI: 0.6358-0.7706 (5000
  stratified bootstrap
  replicates)
```

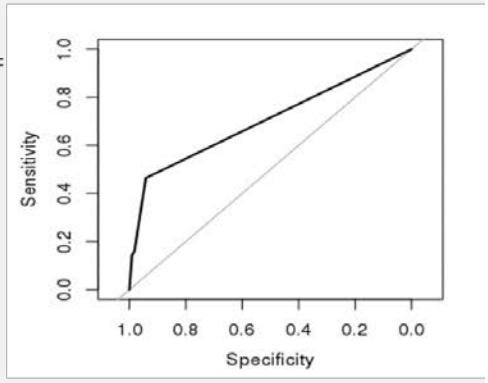



Figure 2.4. Confidence intervals-95%, boot.ci. This is a sample code that was written for confidence interval analysis performed in chapter 4 to determine the effect size of treatment group B (aCGH) against group A (reference). The software package "boot.ci" was programmed to run 5000 replicated for the determination of the final CI. For this analysis, the Area under the curve was 0.7041 with a 95% confidence of 0.6358-0.7706 accompanied by a ROC curve.

With careful consideration of applicable statistical methodologies, I used evolutionary tree (Evtree) predictive modeling analysis for the data analysis in Chapters 3, 5 and 6. I used random forest predictive modeling analysis for data analysis in Chapter 4. All predictive models were

accompanied by ROC curves to determine the overall accuracy of the model along with 95% CI to indicate the effect size of treatment, taking both specificity and sensitivity into account.

# CHAPTER 3

# GRAFT TRANSFER-EMBRYONIC STEM CELLS (GT-ESC) IN SIBLINGS BLASTOCYSTS OF THE MOUSE, *Mus musculus*

## 3.1 Introduction

The first binary cell differentiation in mammalian embryos occurs when their totipotent cells differentiate into either an inner cell mass (ICM) or trophectoderm (TE). This critical action results from the expression of various transcriptional factors activating genes specific to either TE cells or ICM (Ralston and Rossant 2010). Prior to blastulation, one of the most crucial events in mammalian embryonic development is compaction. Mouse blastomeres, following the third cleavage, form a compact ball of cells referred to as a morula (Kelley and Gardner 2017). Each morula consists of inner cells surrounded by a large group of external cells. The compaction is promoted in part by the expression of cell adhesion proteins such as E-Cadherin, which when expressed, form tight junctions between the outer cells of the morula (Li et al. 2009; Maître and Heisenberg 2013; Toh et al. 2015). Most of the external cells become the TE; whereas the inner cells become the ICM. As the embryonic cells continue to develop, blastulation positions the ICM to one side of the TE ring. Upon blastulation, TE cells are bound via E-Cadherins (Alikani 2005). This trans-membrane, calcium dependent, glycoprotein of 120-kDa interacts with the cytoskeleton through catenin proteins (Maître and Heisenberg 2013) enabling a successful adhesion and integration of cells (see Chapter 1). E-Cadherin also regulates epithelial junction formation while associating with catenin proteins (Zaidel-Bar 2013; Palacios et al. 2005; Fleming et al. 2001). The ICM gives rise to the fetus whereas, the trophectoderm cells give rise to extra-embryonic structures such as the placenta, umbilical cord and the tissues of the chorion, which aid in delivery of oxygen and nutrients from mother to the developing fetus (Natale et al. 2017; Sagrillo-Fagundes et al.

2016). The formation of the ICM is, of course, as crucial to live birth as the proper formation of the TE cells. Both cell types are therefore, assessed in *in vitro* fertilization (IVF) procedures.

*In vitro* fertilization is the process, which allows for multiple oocytes from a genetic mother to be inseminated during one treatment creating multiple blastocysts. Blastocysts develop simultaneously in culture, of which one well developed blastocyst may be transferred to the mother's uterus for a presumable pregnancy. The remaining high quality blastocysts may be cryopreserved for future potential pregnancy attempts. It is on the $5^{th}$ day of human embryo development when blastocysts are fully formed and observed *in vitro* and are morphologically assessed. TE and ICM cells differentiate and can be distinguished at this time in development. High quality blastocysts determined to be suitable for uterine transfer or cryopreservation exhibit a well formed TE and ICM group of cells. There are however, blastocysts which develop an exiguous number of TE cells even if the ICM is well developed. Such blastocysts are destined for discard due to low survivability from vitrification and warming (Zhu et al. 2014; Veleva et al. 2013).

To optimize the number of high quality blastocysts produced from an IVF treatment, I have explored whether those blastocysts producing a large number of TE cells can "donate" 8-20 cells to a sibling blastocyst with poor TE health in the form of a cellular graft. This graft would aid the latter sibling blastocyst in continued development, increasing its potential for success and likelihood of selection for cryopreservation to use with future uterine transfers, eventually in human IVF. The purpose of this study, then is to introduce a laboratory technique using blastocysts of the mouse, *Mus musculus*, which takes advantage of the adhesion properties of cells, specifically E-cadherin proteins during micromanipulation. I aimed to validate a technique, which may eventually enable rescuing of poorly growing human blastocysts (from a fate of discard) without

disruption of ICM cells. Specifically, I hypothesized that chromosomally different transplanted trophoblast cells from sibling blastocysts are able to rescue a poor quality sibling blastocyst's development.

## 3.2 Materials and Methods

### 3.2.1 Source of and Description of Embryos

Mouse embryos were purchased from EmbryoTech Laboratories (Haverhill, MA) in cryopreserved straws of 10 or 20 2-cell stage embryos per straw. A total of two batches were used during the study (B2-2016416 and B2-10915). Institutional animal care and use committee (IACUC) approval was obtained for this study, 15019, from the University of North Texas on September 02, 2015 (Appendix D).

### 3.2.2 Thaw and Culture

All cultures were performed in incubators (Sanyo MCO-5M) programmed for 6.2% $CO_2$ and 5.0% $O_2$. Gas values were verified daily by a G100 handheld analyzer (ViaSensor). An example of a calibration curve for this device is presented in figure 3.1. The day prior to embryo thawing, one 35mm Petri dish (Thermo Scientific™ 153066) was prepared with seven-40µl drops of culture medium, G1™ Plus (VitroLife, 10128) for equilibration, in which the embryos will grow for the first 3 days then G2 ™ Plus for days 3-6 (VitroLife, 10132). Each dish was overlaid with 4ml of culture oil (VitroLife, 10029). On the day of thaw, one straw containing either 10 or 20 2-cell mouse embryos was removed from the cryotank and held at room temperature for 2-3 minutes. The straw was then placed in a 37˚C water-bath for one minute to complete thawing. The lower end of the straw was cut to expel cryo-media containing the embryos and upper end was cut

between the plug (appendix A). A rod was used to depress the plug and expel the embryos onto a dish. The embryos were found and rinsed in a 100µl drop of modified human tubal fluid (mHTF) (Sage, ART-1023) supplemented with 10% protein (Irvine, 99193) using an Eppendorf micropipettor. The embryos were rinsed in a second 100µl drop of mHTF then moved to the third 100µl drop of mHTF and allowed to equilibrate for 10 minutes at room temperature. At the end of the thawing period, the embryos were placed in a pre-equilibrated G1 Plus dish of culture medium and left in the incubator overnight. The day of the thaw was day 1 of embryonic development. Embryos were moved to G2 Plus medium on day 2 of culture thru day 6.

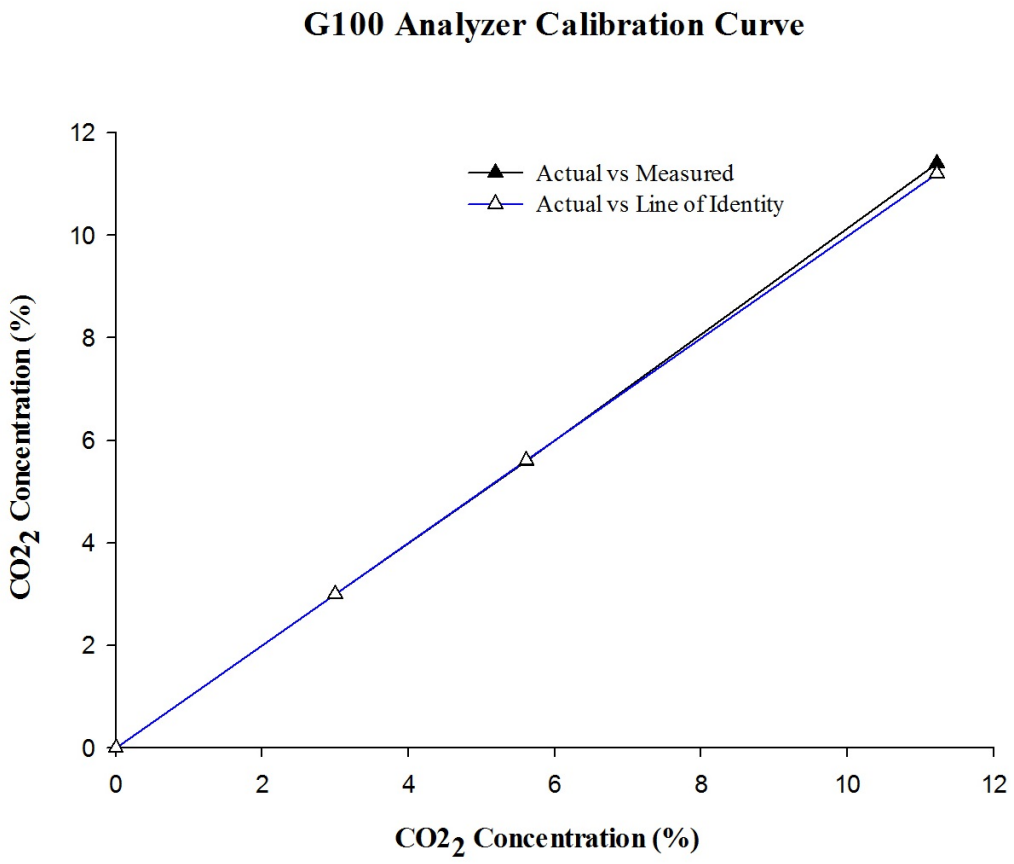



Figure 3.1. Calibration curve. G100 handheld analyzer was verified for accuracy and used to collect all of the daily incubator chamber gas measurements. The line of identity (Blue) is plotted as well as the analyzer measured values against the actual values.

### 3.2.3 Definition of Outcome and Terminology

*Recipient blastocysts* are sibling blastocysts that received a graft. *Donor blastocysts* are blastocysts, which donated either ICM or TE cells to the recipient blastocyst(s) (Note: Recipient and Donor terms are used differently in this chapter as opposed to subsequent chapters, in which donor refers to individual human patients that donated gametes and recipients refers to individual human patients who accepted gametes from donors). *Grafts* are defined as either 8-20 TE or ICM cells that were removed from sibling donor blastocysts and transplanted to the sibling recipient TE cells. *Success* is defined as positively integrated graft cells with the recipient's sibling TE.

### 3.2.4 Technique Overview

An overview of this technique is presented in figure 3.2, in which the top of the figure demonstrates a high quality blastocyst that has a proper inner cell mass (ICM) and trophectoderm (TE) cells. The next two blastocysts have either the ICM or TE cells developed poorly, resulting in blastocyst discard. The proposed technique of micromanipulation of the blastocysts, in which either ICM or TE develops poorly is demonstrated in the lower half of the figure. The first scenario (A) depicts how a good piece of one blastocyst (ICM) could be transplanted into the good piece of a sibling blastocyst (TE). Once performed, it would result in one high quality blastocyst by combining the strong cell types from two poor quality blastocysts. Alternatively, as shown in scenario B, a blastocyst with well developed ICM and TE cells could donate 8-20 TE cells to a sibling blastocyst, which has developed a high quality ICM but a poor quality TE, creating a fair quality blastocyst. Ultimately the number of high quality blastocysts per IVF treatment would be higher, in turn saving blastocysts that would otherwise be destined for discard.

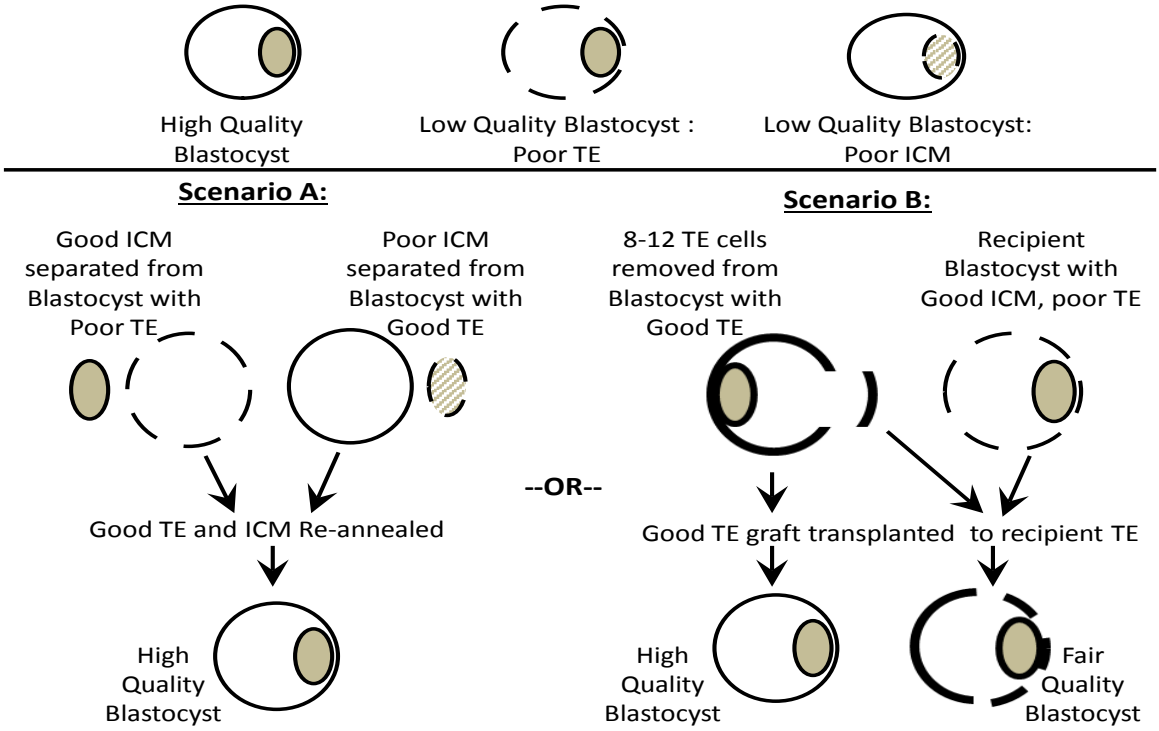



Figure 3.2. Technique overview. The large solid lined circle represents a good quality trophectoderm (TE) in blastocyst and the hashed circle represents a poor quality TE. The smaller oval circles shaded solid gray are representative of good quality inner cell mass (ICM) where as the hashed smaller oval circles are poor quality ICM. A good quality ICM can be grafted from one blastocyst containing a poor quality TE into a sibling blastocyst that has a good quality TE but a poor quality ICM. This allows for the creation of one high quality blastocysts from two poor quality initial sibling blastocysts. Alternatively, one high quality sibling blastocysts can donate 8-20 good quality TE cells to sibling blastocysts, which have a high quality ICM but a poor quality TE creating a fair quality blastocyst. Courtesy: W.W. Burggren, UNT.

### 3.2.5 Study Organization and Grouping of Blastocysts

Modified human tubal fluid (mHTF) medium, a synthetic solution composed of HEPES buffering system with protein supplement, was used to determine if the adhesion success of grafts is higher without disruption of E-Cadherin bonds in blastocysts. Additionally, calcium/magnesium free medium was used during cell grafting procedures to determine if the interim dissociation of E-Cadherin-catenin bonds in the recipient blastocyst, temporarily exposing recipient cells proteins to donor graft cell's proteins, will improve graft adhesion after transplantation and restoration of calcium in the medium. The study included 155 thawed 2-cell mouse embryos. Due to their early

embryonic developmental arrest prior to treatment, 16 embryos were excluded from the study. The remaining 139 embryos at the blastocyst stage were divided in two groups: Group A (n=97) with modified human tubal fluid (mHTF) medium, (Irvine Scientific, 90126) used at treatment; Group B (n=42) with GPGD (VitroLife, 10074) medium used at treatment.

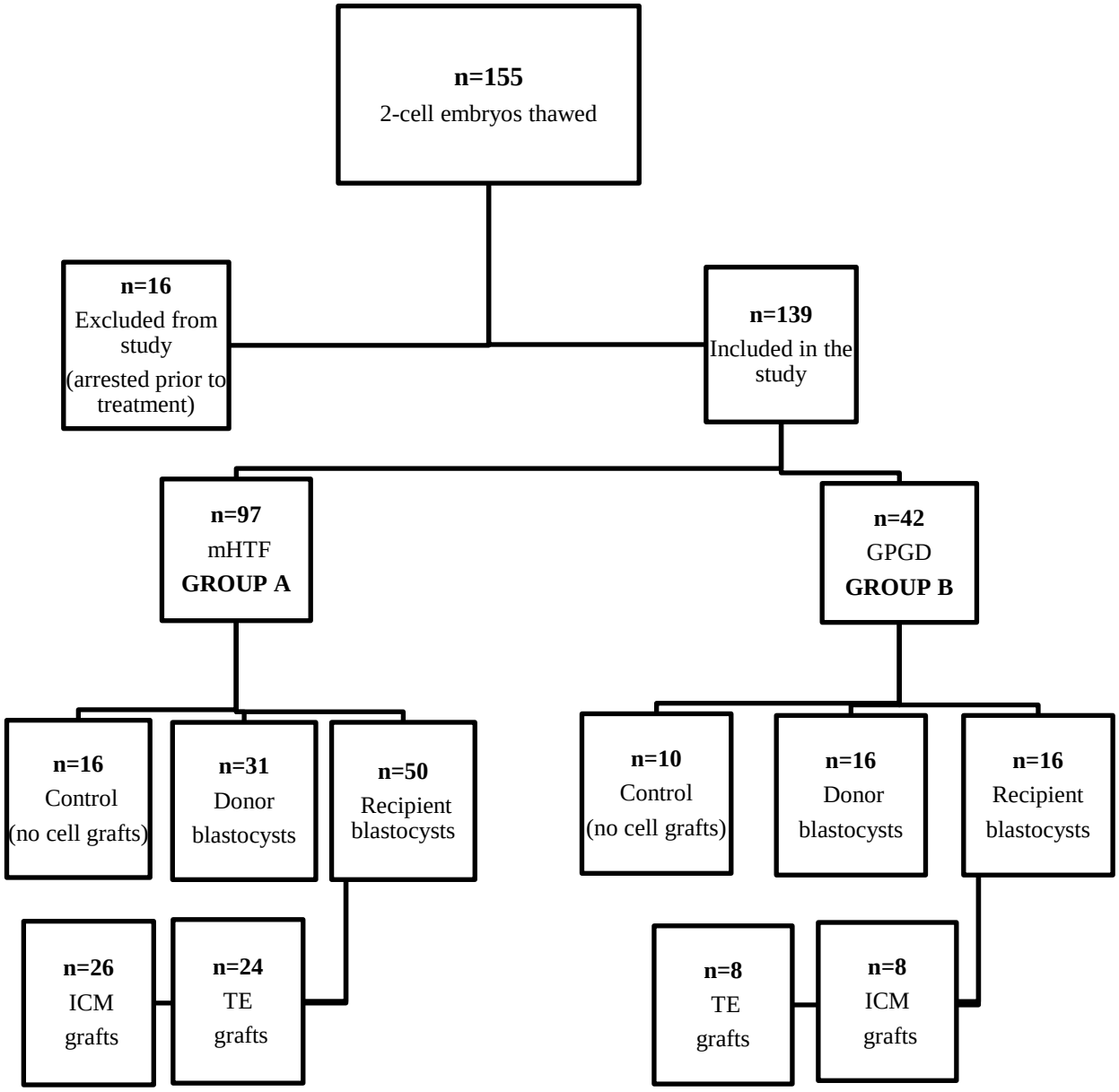



Figure 3.3. Study organization. Control blastocysts were exposed to treatment medium; however they were not exposed to cell ablation via Saturn laser pulses. Mouse blastocysts were organized in two main treatment categories of mHTF medium exposure during the cell grafting procedure or GPGD medium.

The study organization is depicted in figure 3.3. In Group A, 42 total blastocysts were divided into three subgroups: control (n=16), donor (n=31) and recipient (n=50). Blastocysts in group B were similarly divided in three subgroups: control (n=10), donor (n=16) and recipient (n=16). Recipient blastocysts in groups A and B each either received inner cell mass (ICM) grafts (Group A, n=26 and Group B, n=8) or trophectoderm (TE) grafts (Group A, n=24 and Group B, n=8).

### 3.2.6 Blastocyst Cell Grafting

For each round of embryo thaw, the graft transplant was scheduled on day 4 in one half of the treatments and day 5 on the other half to determine if success was stage-dependent. On the scheduled graft transplant day, selected blastocysts based upon grouping were placed in a dish (Falcon, 353655) pre-warmed to 37˚C. The dish consisted of 10 - 10µl drops of either mHTF or GPGD and overlaid with 4ml of culture oil. Cells isolation of grafts, previously described, was performed using the Saturn laser, which consists of 1480nm / 400mW solid state diode laser with a pulse length range 0.005-2.0ms / 5-2000µs and is embedded in a 40X objective. For the procedure, control, donor and recipient blastocysts were all placed in the treatment dish in individual microdrops for isolation, number assignment and tracking. The dish was placed on the inverted microscope (Olympus IX70) for the entire duration of treatment (30-40 minutes), in which all blastocysts were exposed to the treatment solution for the same duration.

#### 3.2.6.1 Control Blastocysts

Control blastocysts neither received nor donated cells to any other blastocyst nor were exposed to the Saturn laser. The viability, blastocoel expansion cell symmetry and granularity of

the control group for both group A and B was assessed one day post procedure. This group was used as a reference for quality comparison of embryos in treatment groups to determine if a decline in blastocysts quality emerged and if so, was the contributing factor the treatment solution or laser exposure.

#### 3.2.6.2 Donor Blastocysts

Blastocysts in the donor group donated 8-20 TE or ICM cells, referred to as grafts, to the recipient sibling blastocysts. To isolate a graft from the donor, each blastocyst was positioned between a holding pipette (VitroLife, 14393) and biopsy pipette (VitroLife, 15123) under the microscope. The pulses from the laser were emitted at 3.67ms (3.9µm pulse width/hole size). The graft was removed from the donor and held inside the glass biopsy pipette.

#### 3.2.6.3 Recipient Blastocysts

Immediately after the graft had been isolated from the donor, the dish was repositioned and the pipettes were introduced to the microdrop containing a recipient blastocyst, which were recipients of the graft cells. To accomplish this, the recipient blastocyst was held against the holding pipette and the zona pellucida was ablated to create an opening of approximately 15µm (figure 3.4A). The biopsy pipette containing the graft was advanced towards the zonal opening and the graft was carefully inserted through the recipient's zona pellucida taking proper precautions that the side of the graft with ablated cells was in contact with the recipient's trophectoderm (figure 3.4B, C). Once the graft was placed in the recipient's zonal region, the pipette was advanced into the blastocyst to promote collapse of the recipient's blastocoel. As the blastocoel collapsed, it created room for the graft to properly position inside its zona pellucida

(figure 3.4D, E). Finally, the biopsy pipette was pressed against the recipient's zona pellucida to promote contact of graft at the site of ablation with the recipient TE. Once the recipient blastocyst began to re-expand its blastocoel and exert pressure on the graft (held in position by the zona), cells were in the optimal position for successful annealation with sibling TE (figure 3.4F). At the end of the procedure, all blastocysts were returned to the G2 Plus culture dish and placed individually in 40µl drops for overnight culture.

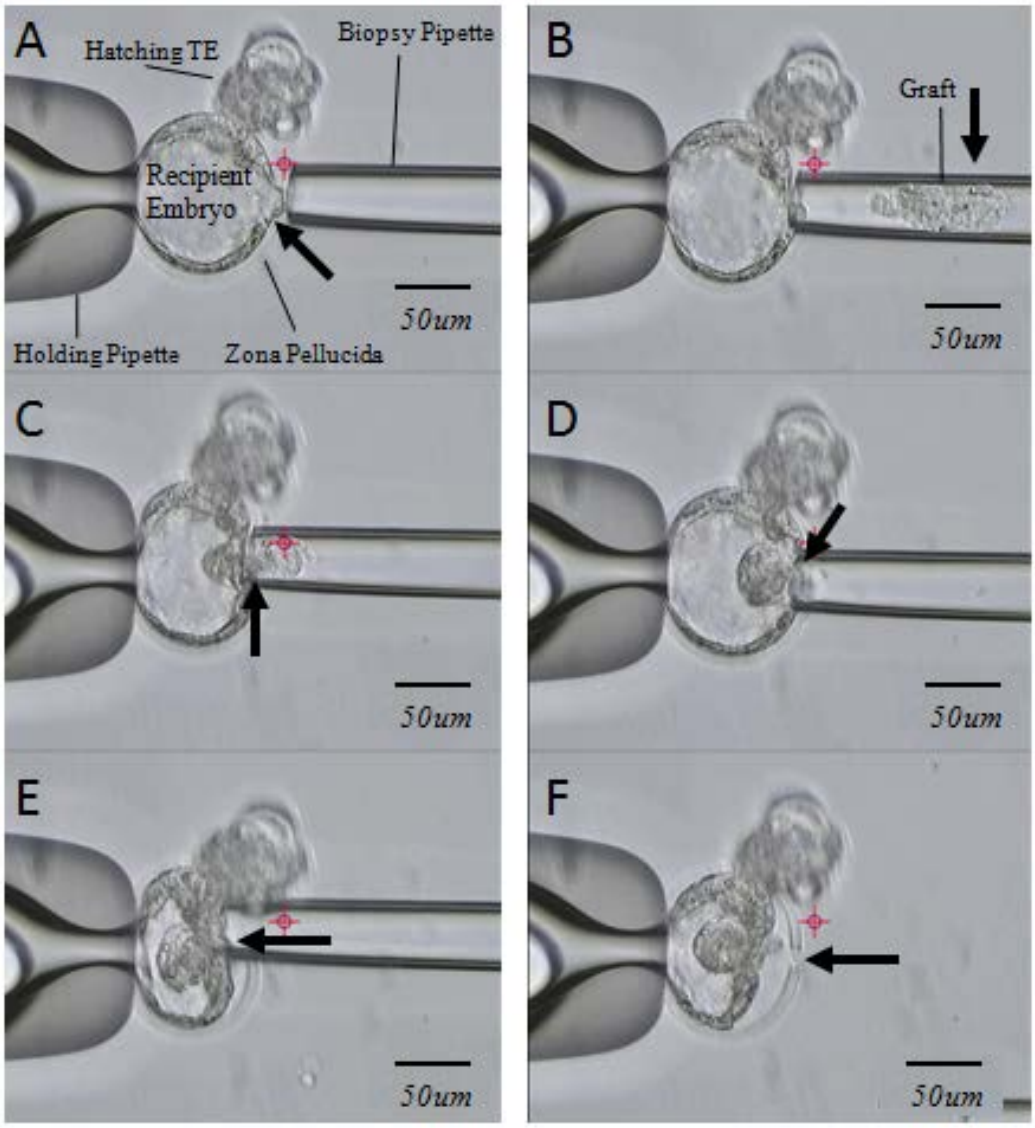



Figure 3.4. Cell grafting procedure. Cell grafts were performed on Olympus IX70, inverted compound microscope using a 40X Saturn laser objective. Grafts were ablated using the Saturn laser embedded in a 40X objective, removed from donor blastocysts and transplanted using a micromanipulation pipette into the zona pellucida of a sibling recipient blastocyst.

### 3.2.7 Assessment of Graft Transplantation Success

One day post treatment, all blastocysts were microscopically assessed, using an Olympus IX70. Measured parameters included viability, blastocoel expansion, distinct inner cell mass compaction, trophectoderm cell symmetry and granularity. Recipient blastocysts were additionally assessed at the graft site to determine if the graft successfully annealed. Next the blastocysts were fluorescently stained for further confirmation of success, which is now discussed.

### 3.2.8 Fixation and Microscopy

Blastocysts were placed in a 20 µl drop of fixative prepared by adding 100 µl of 3.7% formaldehyde (Millipore Sigma, b1040022500) in PBS (phosphate buffered saline) (Fisher Scientific, BP24384) and incubated for 5 minutes at room temperature. Following fixation, the blastocysts were permeablilized using Triton™ X-100 for 5 minutes. This solution was prepared by adding 50µL of Triton X-100 (Sigma-Aldrich, X100-5ml) to 49.95 ml PBS. Blastocysts were then washed twice with block solution formulated with 3% Bovine Serum Albumin (BSA), 40 ml of PBS and 50 µl of Tween 20 (Sigma-Aldrich, P19379) with 2 minutes between washes.

Next, DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Sigma-Aldrich, D9542-1MG, excitation spectrum 358/461nm) solution was prepared by diluting 1 µl of 0.5 mg/ml DAPI in 100 µl of block solution. The nuclei/DNA were stained by incubating the blastocysts in DAPI solution for 5 minutes. The blastocysts were then rinsed twice in block solution of 100 µl drops with two minutes in between each rinse. For examination, blastocysts were placed in 2 µl drops of stock solution on a glass slide. After 5 seconds, a drop of Prolong-Gold anti-fade mountant (Fisher Scientific, P36934) was expelled on the blastocysts and covered with a glass cover slip. Slides were held at 4˚C for a maximum of 2 days until examination. Laser scanning confocal microscopy

was carried out with Zeiss LSM710, laser scanning confocal microscope (Carl Zeiss, Inc, Thornwood, NY, USA), equipped with a 25 mW 405 nm diode laser, a 25 mW multi-line Argon laser (458, 488, and 514 nm), a 20 mW 561 nm DPSS laser, and a 5mW 633 nm HeNe laser. The images are presented as single 4μm thick optical sections.

### 3.2.9 Data Analyses

Morphologic assessment was performed of all blastocysts using the SART grading system (Heitmann et al. 2013). All blastocysts were assessed for expansion of the blastocoel, trophectoderm cell symmetry and granularity. Additionally, recipient blastocysts were assessed for graft integration and adhesion. A decision tree construction was performed with the evolutionary tree algorithm (Evtree) using R software (Grubinger et al. 2014; Fan and Gray 2005) (see Chapter 2). The receiver operating characteristic (ROC) curve analysis, which illustrates the Evtree's diagnostic ability as the discrimination threshold is varied for the statistical computation (Robin et al. 2011) was performed (see Chapter 2). This curve calculates the rate of sensitivity given the optimal specificity. With both specificity and sensitivity computed, an overall accuracy of the model was calculated.

## 3.3 Results

### 3.3.1 Microscopic Assessment One Day Post Graft Transfer

Upon examination, most recipient blastocysts demonstrated one of two conditions: either strong annealation of TE cells to sibling grafts or failure to anneal with the graft where the graft either moved away from the recipient or was attretic (figure 3.5A-C). In figure 3.5A, graft cells were grouped together and initiated a blastocoelic cavity formation separate from the recipient.

Additionally, there were blastocysts that successfully merged with the grafted cells and continued development (figure 3.5D-F). In these images, the black arrows show the location of annealation between the recipient TE and sibling graft. Figure 3.5D demonstrates two hatching points in the recipient zona pellucida. The TE cells hatching from the bottom left belong to the recipient whereas the cells herniating from the top left are the grafted cells identified by the black arrow (successful cell annealation).

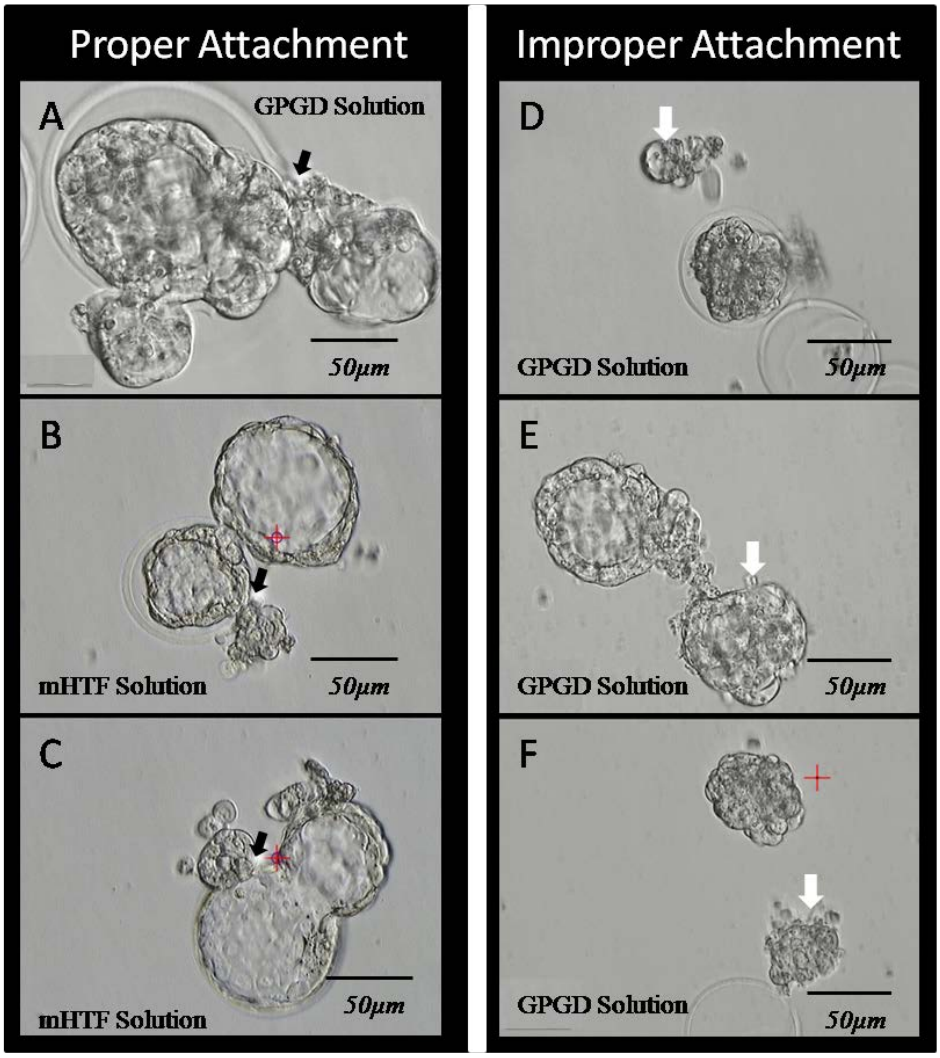



Figure 3.5. Graft success evaluations**.** Representative blastocysts of successful versus unsuccessful graft adhesion Recipient blastocysts were assessed one day post graft procedure for evaluation of successful graft integration in sibling TE cells. Images are labeled to indicate which solution was utilized during procedure. Successful proper attachments (A-C) are identified with black arrows. Improper attachments of the grafts (D-F) were identified as isolated group of cells from the developing recipient blastocyst and are labeled with white arrows.

3.3.1.1 Treatment of Graft Transfer on Day 4

1) *mHTF Solution (group A):* Representative day 5 blastocysts are shown in figure 3.6A-B. Control blastocysts exhibited full expansion of blastocoel, symmetry of TE and distinct compaction of ICM. Recipient blastocysts (figure 3.6B) also showed full expansion of blastocoel on day 5 of development, symmetrical cell pattern present throughout the TE and distinct compacted ICM. Additionally, the graft cells were apparent and site of annealation was clearly observed with a majority of cells at the ablation site in contact and integrated with the recipient TE. No granularity of TE was observed.

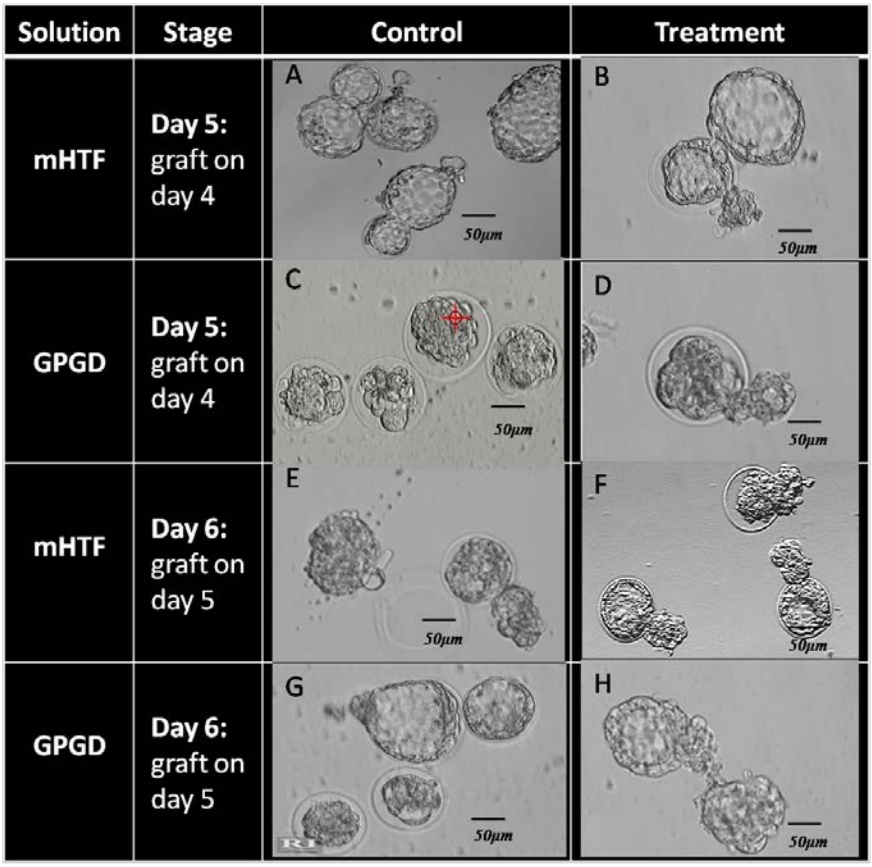



Figure 3.6. Blastocyst qualities by treatment. Blastocysts evaluations were performed one day post graft transplant and exposure to treatment solution. Representative images of blastocyst qualities observed on day 5 of development post treatment on day 4(A-D). mHTF treatment group on day 4 were overall the highest quality on day 5 (A, B). Blastocysts showed overall the lowest morphology with exposure to GPGD on day 4 (C, D). Blastocysts on day 6 exposed to mHTF (E) demonstrated expansion of the blastocoel, symmetry throughout the TE and defined cell borders (F, H). However, full expansion was not observed in this group (G-H).

2) *GPGD Solution (group B):* Control blastocysts on this day showed increase in cell granularity, blastocoelic cavity collapse and a highly inconsistent and asymmetrical pattern of trophectoderm cells. (figure 3.6C). Recipient blastocysts on day 5 similarly showed profuse collapse of the blastocoel within and outside of the zona pellucida accompanied by increased granularity in cell morphology (figure 3.6D). TE cells were largely asymmetrical on the recipient blastocyst. Graft cells, although identifiable, did not demonstrate strong integration with recipient TE cells. Due to blastocoelic collapse, cell borders were difficult to distinguish for evaluation of cell viability.

3.3.1.2 Treatment of Graft Transfer on Day 5

1) *mHTF Solution (group A):* Control blastocysts were fully to partially expanded, displayed symmetrical TE cells, ICM was compact and identifiable and cell granularity was absent (figure 3.6E). The overall expansion patterns of the recipient blastocysts, likewise, ranged from fully expanded to partially expanded blastocoelic cavities (figure 3.6F). TE cells were symmetrical and ICMs were compacted and distinct. Clear and concise cell borders were observed throughout the blastocysts and no cell granularity was observed. Grafts were identifiable and demonstrated annealation, when successful, of all cells at the ablation site in contact and integrated with the recipient's TE. Little to no degeneration of cells on grafts at the ablation site was noted.

2) *GPGD Solution (group B):* Control blastocysts exhibited partial expansion of the blastocoel. Figure 3.6G shows four control blastocysts, of which three are partially expanded and one shows complete collapse of the blastocoelic cavity representative of morphology for this group. Symmetry of the TE is moderate in blastocysts with at least partial blastocoelic expansion. For recipient blastocysts, the morphology on day 5 similarly varied slightly ranging from partial

expansion of the blastocoel to complete collapse (Figure 3.6H). Full expansion of the blastocoel was absent in all of the recipient blastocysts. Although present, granularity was slightly less evident in the recipient TE cells compared to graft transfer one day earlier in this solution. Due to the partial to full collapse of the blastocoel, the cell borders were less prominent. Grafts were apparent, but the overall annealation resulted in 4-6 cells integrated in the recipient's TE with the remaining cells at the ablation site degenerating and failing to anneal.

### 3.3.2 Nuclear Staining Assessment

Single optical sections of recipient blastocysts were analyzed for the interaction between the nuclear pattern of the graft cells and the recipient trophectoderm cells. Nuclei stacked linearly against the recipient TE indicated failed integration of grafted cells. A netted pattern of graft cell nuclei with continuous extension in recipient TE would, however, indicate a positive annealation and integration with the recipient sibling blastocyst. Figure 3.7A is representative of a recipient hatching blastocyst that was successful in incorporating the sibling graft with its TE. The white arrow on the left of the image points to a linear nuclear pattern of the graft that is folded along one side of the sibling TE (no integration on this part of the graft) and the gray arrow on the right of the image shows the area of the graft that has successfully integrated with the recipient blastocyst trophectoderm, displaying a netted pattern of nuclei extending from the graft cells and continuous throughout the recipient cells. Figure 3.7B shows a blastocyst expanded within its zona pellucida with failed attachment of the graft. The arrow is pointing to the graft which has failed to integrate, demonstrated by the stacked linear nuclear pattern along the side of recipient TE cells. The netted integrative pattern confirming integration is absent.

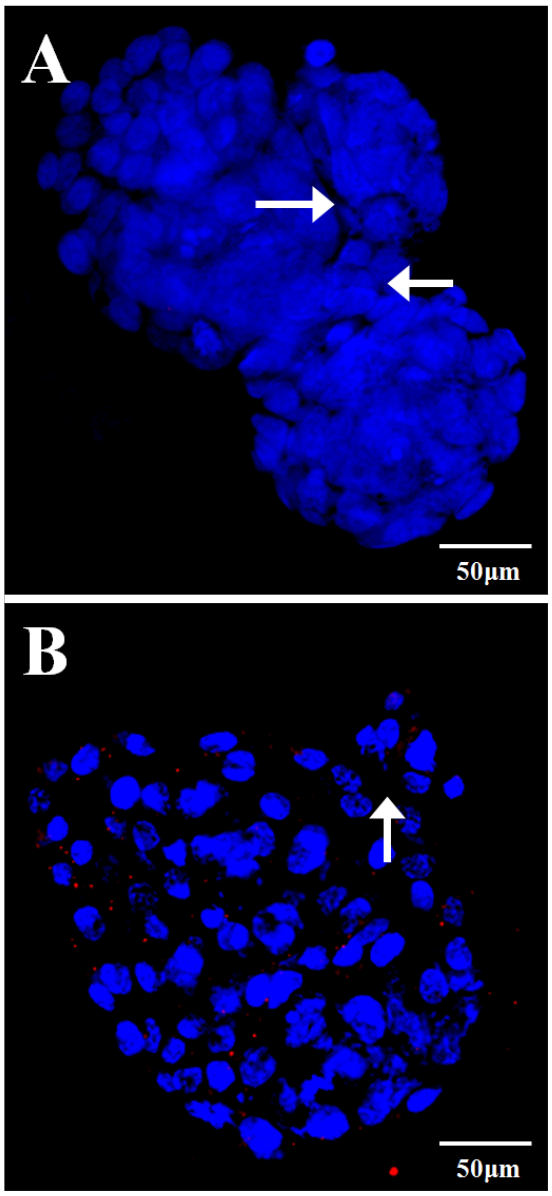



Figure 3.7. Fluorescent nucleic acid staining. Blastocysts that successfully accepted the sibling graft demonstrated a netted integration of cell nuclei that was continuous from the graft throughout the recipient TE (A) depicted by the gray arrow. When the graft was unsuccessful or the portion of the graft cells not directly integrated with the sibling TE appeared as a stack linear organization of cell nuclei (B) depicted by the white arrow.

### 3.3.3 Evtree Analysis for Graft Cell Adhesion

The success for each recipient blastocyst was measured by a confirmed attachment of the graft, whereas all other outcomes were labeled as no attachment (including attretic grafts or attretic blastocysts). Using this measure as the final outcome a decision tree analysis was performed in R

software using Evtree (see Chapter 2) (Grubinger, Thomas, Achim, and Pfeifer, Karl-Peter 2014). According to the prediction model (figure 3.8), the type of cells in the cell graft (TE or ICM) was not a factor that impacts outcome and so was not displayed on the final tree. The medium, in which the blastocysts were placed during the graft procedure had the greatest impact on outcome (figure 3.8, node 1). Day of the graft transplant was the second factor which determined outcome success (node 2).

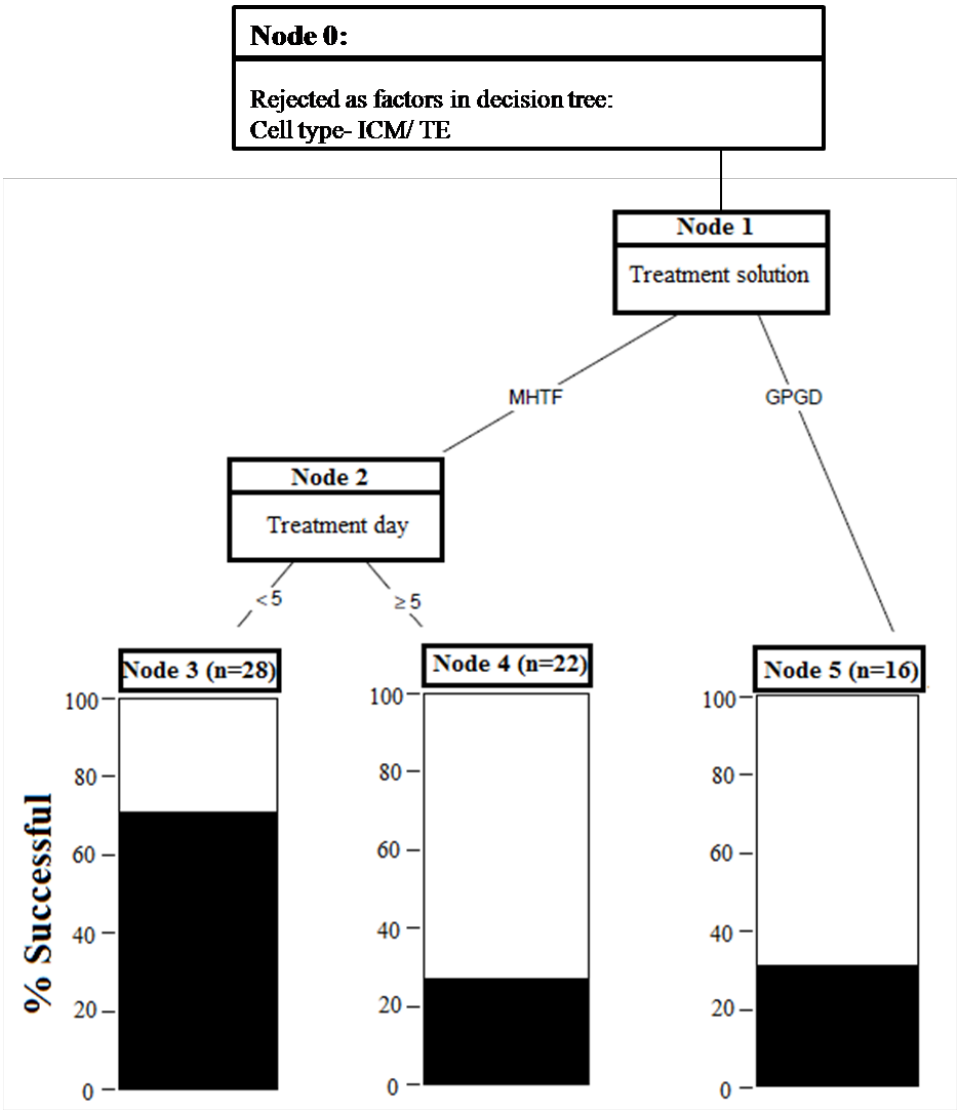



Figure 3.8. Evtree for graft cell adhesion. This prediction model was determined by Evtree in R software. Final graphed nodes depict highest predictive success of graft adhesion when transplantation is performed on day 4 using mHTF as procedural medium (Node 3) with approximately 70% success.

Graft procedures using mHTF had the highest success (70%) in graft integration with the recipient sibling trophectoderm when grafts are transplanted on day 4 of development (node 3, n=28).When graft transplants were performed on day 5, success decreased to ~25% (node 4, n=22). Transplants performed in GPGD had 30% success in graft attachment to the recipient's trophectoderm (node 5, n=16).

### 3.3.4 Accuracy Determination of Evtree Analysis

Evtree analysis yielded an accuracy of 71% with an error rate of 29% (figure 3.9). The ROC curve had an area under the curve (AUC) of 0.71 with 95% confidence interval (CI) of 0.6-0.8, indicating that any final node of the decision tree results in 10% greater success than chance alone as the lowest measure and up to 30% greater success than chance alone at its highest success.

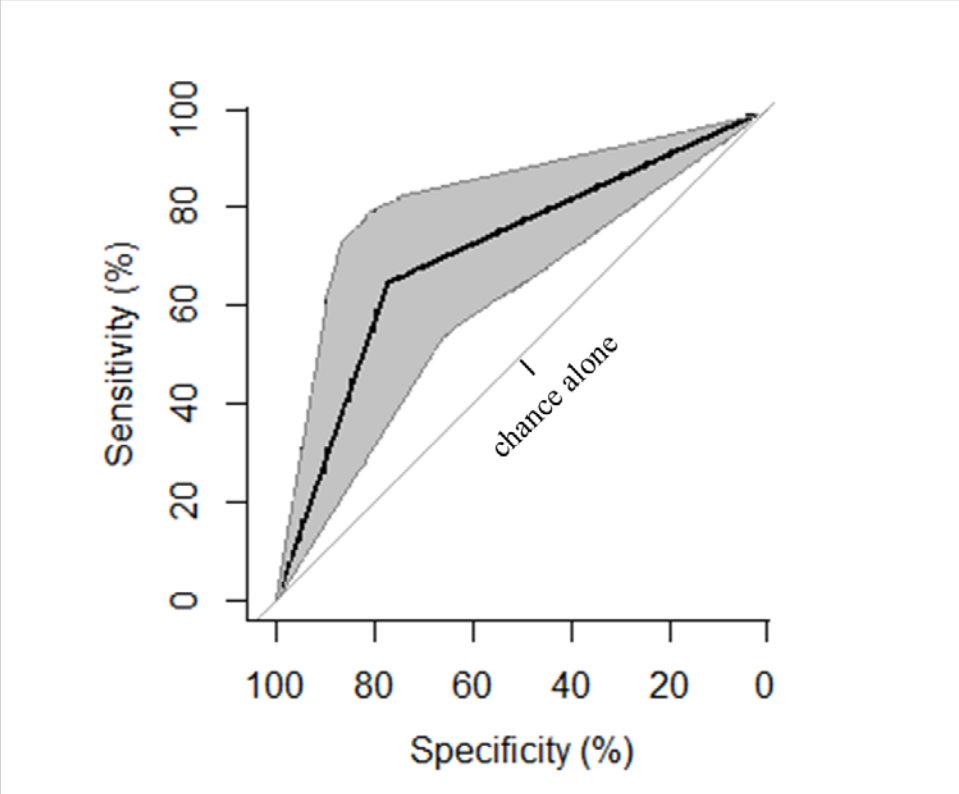



Figure 3.9. Receiver operator characteristic curve: cell adhesion. The receiver operator curve for Evtree analysis of graft cell adhesion. True positive rate (sensitivity) is plotted against the false positive rate (1-specificity). The vertical lines represent 95% confidence intervals.

## 3.4 Discussion

Numerous techniques offer exclusion criteria to aid in the selection of one superior blastocyst from a group of sibling blastocysts for uterine transfer to optimize pregnancy outcome. These techniques include morphology assessment, preimplantation genetic screening and morphokinetic assessments with tools such as time-lapse microscopy (Fragouli and Wells 2012; Capalbo et al. 2014; Mumusoglu et al. 2017). There are limited inclusion methodologies for individuals at risk of poor outcome, such as no high quality blastocysts produced per treatment, which may incorporate poor quality embryos. The technique of the present study provides an inclusion method for IVF, increasing the number of high quality blastocysts created per treatment from graft donation of sibling blastocysts.

### 3.4.1 Stage-Dependent Graft Success

In the present study, on days 5 and 6 of development, the blastocysts were expected to demonstrate full blastocoelic cavity expansion, concise visible cell boarders, compacted and distinct inner cell mass cells, symmetry in trophectoderm cells and no granularity in cell morphology. The grafts were expected to show the above mentioned morphology as well as clear distinct incorporation of annealed cells into the recipient blastocysts' TE for treatment groups. The adhesion of the trophectoderm cells in blastocysts was assessed for successful integration of donor cell grafts from sibling blastocysts by microscopy and fluorescent nucleic acid staining. The resulting images suggest that successful graft integration is stage-dependent. Grafts transplanted on day 4 of development (day after morula stage) had the combination of highest rate of successful graft adhesion and stage appropriate growth of full blastocoel expansion with symmetrical, agranulated cell morphology in the mHTF medium. Trophectoderm cell junctions in the

blastocysts, when not disturbed during development (removing calcium from the medium) provides optimum conditions for annealation and incorporation of sibling graft.

Both mHTF and GPGD media treated control blastocysts on day 4, displayed the same pattern of morphology on day 5 as the treatment group of recipient blastocysts, which also displayed incorporation of grafts. However, blastocysts treated with GPGD failed to re-expand their blastocoel indicating a loss of functionality essential for continued development. Exposure to GPGD at this stage has contributed to this difference in morphology and growth potential, confirming GPGD's contribution to the blastocysts' inability to completely re-expand post-treatment. Most conspicuous is the presence of severely granulated cell morphology observed in blastocysts treated with GPGD on day 4. This morphology was absent in blastocysts treated with mHTF at the same stage indicating the removal of calcium in the blastocyst's environment caused a negative effect. This is contrary to the expected temporary disruption by GPGD of E-Cadherin to catenin bonds. In human blastocysts, Alikani (Alikani 2005) showed that poorly developed TE cells demonstrate a perturbed distribution of E-Cadherin, which became more erratic or absent as the blastocyst continued growth. This inability of the poor quality TE to properly redistribute E-Cadherin may plausibly explain their inability to recover post exposure to GPGD in the current study.

### 3.4.2 Role of Trophectoderm Junctional Proteins

The trophectoderm and inner cell mass communicate through gap and tight junctions as well as other structures (Fleming et al. 2000; Ren et al. 2016; Watson and Barcroft 2001). However, blastocysts with abnormal morphology have both altered expression and function of these junctional proteins (Hardy et al. 1996). Images obtained in the current study indicate that

obstruction of these junctions, particularly in blastocysts with fewer TE cells result in lowered ability of the blastocyst to properly redistribute and continue expression of these essential proteins. Earlier stage blastocysts on day 4 are able to recover in the form of partial blastocoelic expansion whereas later stage blastocysts demonstrated loss of this ability. When grafts were transferred on day 5, exposure to GPGD was delayed by one day and partial expansion of the blastocoel was observed on day 6. The cell adhesion provided by E-Cadherin, expected to increase in number of bonds with continued cell divisions, establishes the tight junctional seal and maintains the polarized Na/K-ATPase distribution essential to blastocoelic expansion (Watson 1992; Harris and Tepass 2010). Therefore, delaying the graft procedure and exposure of blastocysts to GPGD to day 5 provided time for further development and maturation of trophectoderm cells in the blastocyst, conceivably explaining the partial recovery of the blastocoel.

A major reason blastocysts fail to implant is the improper formation of trophectoderm cells, evident from low cell numbers and cell asymmetry as examined prior to uterine embryo transfer in human IVF (Alfarawati et al. 2011; Capalbo et al. 2014). Blastocysts of this low quality are, therefore, not suitable for cryopreservation due to lower implantation rate and survivability from the cryopreservation procedure (Capalbo et al. 2014). Currently these blastocysts are discarded in IVF treatments. The trophectoderm cells support the developing fetus during pregnancy and have differentiated from the fetal cells or the ICM, an irreversible action (see Chapter 1).

### 3.4.3 Implications for Human IVF

In light of our findings I suggest that sibling trophectoderm grafts, chromosomally different from the recipient trophectoderm, can improve development of poor quality blastocysts and possibly support pregnancy by contributing to extra-embryonic structures, previously described

(see Chapter 1). Donation of 8-12 TE cells to a sibling blastocysts will not violate any genetic integrity of the developing fetus, therefore providing aid in implantation and placental support of its sibling only. This is may be compared to a blood transfusion where the donation of blood cells of one genetic person can aid in sustaining life of a genetically different person, however, not altering the blood recipient's ability to express their own genome.

Eventual use of graft transfer embryonic stem cells (GT-ESC) in human IVF is expected to demonstrate a 10-15% increase in high quality blastocyst production per treatment in poor prognosis patients. This inclusive technique will decrease excessive administration of ovarian stimulation medication used to produce a large number of mature oocytes during IVF treatments to increase odds of pregnancy outcome. Lower gonadotropin use will decrease treatment costs per patient and lower incidences of ovarian hyperstimulation syndrome, supporting advancement of the field of reproductive medicine. This is especially important for patients who are diagnosed with diminished ovarian reserve (DOR) or preliminary ovarian failure (POF) whose treatment often does not produce a high number of ovarian follicles and subsequently, lower numbers of high quality blastocysts per round of IVF. Although a pregnancy can be achieved with just one blastocyst, failure to produce additional blastocysts available for cryopreservation for future use, leads to suboptimal use of patient financial resources as well as increased emotional stress.

## 3.5 Conclusion

Analysis of the images in this study support the explanation that failure of proper blastocoel formation is highly associated with the Na/K-ATPase activity or lack thereof (Câmara et al. 2016; Lafond et al. 1991). The implications of temporary disruption of junctions as well as role of Na/K-ATPase in the incorporation of high quality TE cells from sibling blastocysts are therefore well

worth investigating. This is especially important to the application of our technique to human IVF, particularly individuals with poor prognosis such as patients diagnosed with DOR or POF who would most benefit from this method. Especially for this group of patient's the availability of graft transplants to sibling blastocyst may be the most successful option for a live birth. The eventual clinical application will require evaluation of the recipient blastocyst's integrity prior to consideration for uterine transfer, i.e., preimplantation genetic screening. The potential pairing of this technique and PGS may lead to revolutionizing the practice of IVF.

# CHAPTER 4

# EFFECTIVENESS OF PRE-IMPLANTATION GENETIC SCREENING USING NGS, aCGH AND SNP MIRCROARRAY FOR IN-VITRO FERTILIZATION TREATMENTS

## 4.1 Introduction

Aneuploidy, the presence of unbalanced or abnormal number of chromosomes in cells, is more prevalent in individuals with recurrent pregnancy loss (Atasever et al. 2016; Hatirnaz et al. 2017; Hodes-Wertz et al. 2012) and unexplained implantation failure (Hatirnaz et al. 2017). Aneuploidy is one of the most common form of genetic error found in zygotes and early embryos (Handyside et al. 2012; Gleicher et al. 2014; Dahdouh et al. 2015). To screen possible defects, pre-implantation genetic screening (PGS) of embryonic cells prior to uterine transfer is recommended in IVF treatments to reduce failed attempts at embryo implantation and pregnancy losses. Since the early 1990's chromosomal screening methods have been available to IVF laboratories to detect genomic anomalies in developing embryos (Gleicher and Orvieto 2017; Speleman et al. 1992). Since the development of PGS, the primary focus of IVF has been to maximize pregnancy outcome via screening and prevention of aneuploid embryos transferred (Delhanty et al. 1993; Munné et al. 1993; Handyside 2013). Despite this relatively long history of genetic screening, there remains a lack of consensus on the most effective method of screening embryos for IVF treatments. Approximately 5% variability exists in efficiency of array comparative genomic hybridization and next generation sequencing techniques (Aleksandrova et al. 2016). aCGH was advocated as the method of choice for screening of embryos, specifically for women of advanced maternal age (Handyside et al. 2012).

Array comparative genomic hybridization (aCGH) has been successfully used in IVF worldwide as a means to screen all 23 pairs of chromosomes in developing embryos (Adler et al.

2014; Yang et al. 2013; Greco et al. 2014). While aCGH was proven successful and reliable, other techniques have been introduced to further improve pregnancy outcome post embryo biopsy. Single nucleotide polymorphism array (SNP array), which distinguishes between homogeneity and heterogeneity, is successfully used in IVF (Treff et al. 2010; Lathi et al. 2012). Changes in chromosome copy number and deletions or duplications of part or whole chromosomes can also be identified (Li et al. 2014). An added benefit to this technique compared to aCGH is that it can detect uniparental disomy, confirm parentage and detect some mosaic cell lines, embryos with more than one population of cells with different genotypes.

The most current screening method, next generation sequencing (NGS), was introduced to IVF in 2007 (Goodwin et al. 2016; Yang et al. 2015; Wells et al. 2014). This technology is high throughput and can detect mosaic lines in samples, translocations and triploidy. Despite the availability of the improvement in read depth, there is no confirmation that NGS methodology improves implantation and pregnancy rates overall compared to aCGH or NGS. The description of each PGS technique, performed by reference laboratories, is discussed next.

### 4.1.1 PGS Methodologies

*aCGH*: Whole genome amplification of cells was performed by Reprogenetics Laboratory (BlueGnome, Breaks House Mill Court, Great Shelford, Cambridge, CB22 5LD, UK) using 24Sure. This array is based on bacterial artificial chromosomes (BACs) with greater than 5000 DNA clones that are not documented to occur in disease regions, which covers 30% of the entire genome. Once the products are amplified, the test sample is hybridized with the control sample on the array. Both the control and test sample are differentially labeled with florescent probes and the ratios are interpreted. Results are reported as euploid, monosomy of certain chromosome(s),

trisomy of certain chromosome(s), or complex abnormal (multiple chromosome duplications or deletions).

*NGS-targeted amplification regions*: Developed by the Johns Hopkins PGS team (Hallam et al. 2014), targeted next generation sequencing technique differs from whole genome amplification NGS in that "targeted" regions of the genome are amplified. Targeted NGS by EmbryVu (GoodStart genetics laboratory, Cambridge, MA) lowers the overall cost of the test by only amplifying regions to be read and tested. A single PCR primer amplifies the repetitive regions occurring on every chromosome. These regions have enough similarities for one primer to hybridize to all regions. However, these same regions have enough variance that they can be realigned back to their correct corresponding chromosome. The proportions of the repeats that align back to the chromosomes are used to then assess for aneuploidy.

*SNP array:* Samples are processed using Illumina HumanCytoSNP-12 DNA BeadChips (Natera laboratory, San Carlos, CA) and results are interpreted using an informatics-based algorithm referred to as Parental Support (Johnson et al. 2010; Shi et al. 2013). The software references parental genotypes and distinguishes between balanced and unbalanced chromosome copy number, including uniparental disomy. The Parental Support algorithm statistically calculates the confidence scores to determine the accuracy of the genotype detected in the test sample. SNP array contains small fragments of the reference human genome, for which there are known multiple alleles (Adams et al. 2012; Schroeder et al. 2013). Each allele representation is present on the array and each position corresponds to a specific locus. The DNA from blastomeres is hybridized to the array, in which samples with allele A bind specifically to that allele on the array.

Each PGS screening technique presents benefits over others and gives options to individuals that present with infertility (table 4.1).

Table 4.1. Comparison of preimplantation genetic screening techniques

| PGS Technique | Description | Cost | Processing Time | Pros | Cons | Comment |
|---|---|---|---|---|---|---|
| aCGH array comparative genomic hybridization | Test and reference DNA are labeled with different fluorescence probes and bind to complementary DNA on a slide. A 1:1 ratio results in a euploid interpretation. More of one fluorescence results in a gain/loss interpretation and determination of aneuploidy. | $225/ read | 1-7 days | • May detect duplications, deletions and unbalanced translocations<br>• More expensive but faster than SNP | •Requires whole genome amplification (WGA)<br>• Cannot detect Uniparental disomy or triploidy<br>• No simultaneous PGD can be performed | Balanced cost, time and comprehensiveness |
| SNP Array Single nucleotide polymorphism array | Reference chip contains DNA SNPs (single nucleotide polymorphisms) which test sample binds to. A 1:1 ratio interprets as euploidy; gain/loss interprets as aneuploidy. | $234/ read | 7-14 days | •Can be done with parental support to compare allele patterns of parents to offspring, Uniparental disomy can be detected.<br>•Parentage can be confirmed<br>•Detects triploidy and haploidy<br>•Greater resolution than aCGH | •Higher cost than aCGH<br>• Requires WGA<br>• Requires screening of parent DNA for parental support algorithm, added costs | Identifies the source of aneuploidy, maternal/paternal. Beneficial when inheritance patterns matter |
| NGS Next generation sequencing | Sample DNA is fragmented and tagged cDNA library is created. Complementary nucleotides are added and incorporated, in which a sequencer can detect a labeled fluorescence (Illumina HiSeq). Resulting sequences are aligned to reference genome and chromosome copy numbers are calculated. | $150 /read | 1-7 days | •Can detect small duplications/deletions, mosaicism, translocations and triploidy<br>•Simultaneous PGS/PGD can be performed<br>•Can detect sequence variability <5MB<br>•Targeted NGS is possible, WGA not required | •WGA required for non-targeted NGS<br>•Read depth can be adjusted by laboratory<br>•Small deletions/duplications of unknown significance labeled as aneuploid<br>•Targeted version will not detect abnormalities outside targeted area. | Detects mosaicism and gives detailed information on small sequence variations. Greater read depth. |
| None | Embryos selected for transfer based on morphology | n/a | n/a | •Fresh transfers are possible | •May need multiple embryos for transfer to overcome aneuploidy<br>•Increased miscarriages and risk of viable aneuploid gestation | Lower financial burden on patients |

The current study evaluates the pregnancy and implantation rates of NGS screening compared to aCGH and SNP-microarray screening. The objective of this study is to determine

which of the three PGS techniques result in higher pregnancy and fetal viability rates. I hypothesize that SNP array technique, which offers a high sensitivity along with determination of parental origin, is superior to NGS or aCGH in achieving the highest success rates for IVF treatments with transfer of euploid embryos. There is limited information on the efficiency of NGS in a clinical setting for IVF patients, so this study also provides additional information on the acceptability of NGS for clinical use in IVF patients.

## 4.2 Materials and Methods

### 4.2.1 Source of Embryos

This study is a retrospective and prospective analysis of treatments of human patients performed at a single clinic, Dallas IVF, referred to as "the Center[5]." The observational data includes all frozen embryo transfer (FET) treatments from a two year period (January 1, 2015 to December 31, 2016) that fit specific inclusion criteria (discussed below). The data for treatments included in this study were retrieved using an electronic database management system, in which the data were de-identified to remain in compliance with Health Information Portability and Accountability Act (HIPAA).

### 4.2.2 Standard Patient and Embryo Treatment

For all screening groups, individuals underwent controlled ovarian stimulation using gonadotropins followed by transvaginal oocyte retrieval. Individual dosing and all medical procedures related to the *in vitro* fertilization IVF treatment were performed by licensed and board

[5] *Dallas IVF* is a registered member of the American Society of Reproductive Medicine and a Society of Assisted Reproductive Technology. The facility is accredited by College of American Pathology (CAP), Clinical Laboratory Improvement Amendments (CLIA) and is registered with the Food and Drug Administration (FDA).

certified physicians. I performed all *in vitro* fertilization procedures with assistance from members of the Center's embryology team to include: oocyte collection and trimming, culture maintenance, insemination of oocytes, daily embryo checks, trophectoderm biopsy, blastocyst vitrification, blastocyst warming and assistance in uterine blastocyst transfer. Oocytes were inseminated using partner's sperm. All embryos were cultured for a period of 5-6 days, at which they were cryopreserved. All uterine embryo transfers were from warmed blastocysts.

### 4.2.3 Groups by Screening Method

A total of 402 FET treatments were included in the study. Treatments were segregated into 4 groups in accordance to the PGS technique used (Figure 4.1). Group A included 223 treatments, in which 305 of the total embryos transferred lacked the embryo biopsy procedure, but nonetheless underwent cryopreservation in the fresh IVF treatment followed by warming and transfer in the IVF treatment referenced. This group was used as a reference to evaluate the outcomes of the groups with pre-implantation genetic screening methods. The ploidy status of embryos in group A was unknown at embryo transfer for implantation. In groups B, C and D embryo biopsies were performed 5-6 days post oocyte fertilization during the initial *in vitro* fertilization treatments and the embryos were subsequently cryopreserved and biopsies were screened by a third party laboratory. Group B included 56 FET treatments consisting of 71 euploid embryos screened using aCGH analysis (Reprogenetics laboratories, LA). Group C included 50 FET treatments consisting of 63 euploid screened using NGS analysis (EmbryVu, MA). Group D included 73 FET treatments consisting of 97 euploid embryos screened using SNP microarray analysis (Natera, CA) (Figure 4.1).

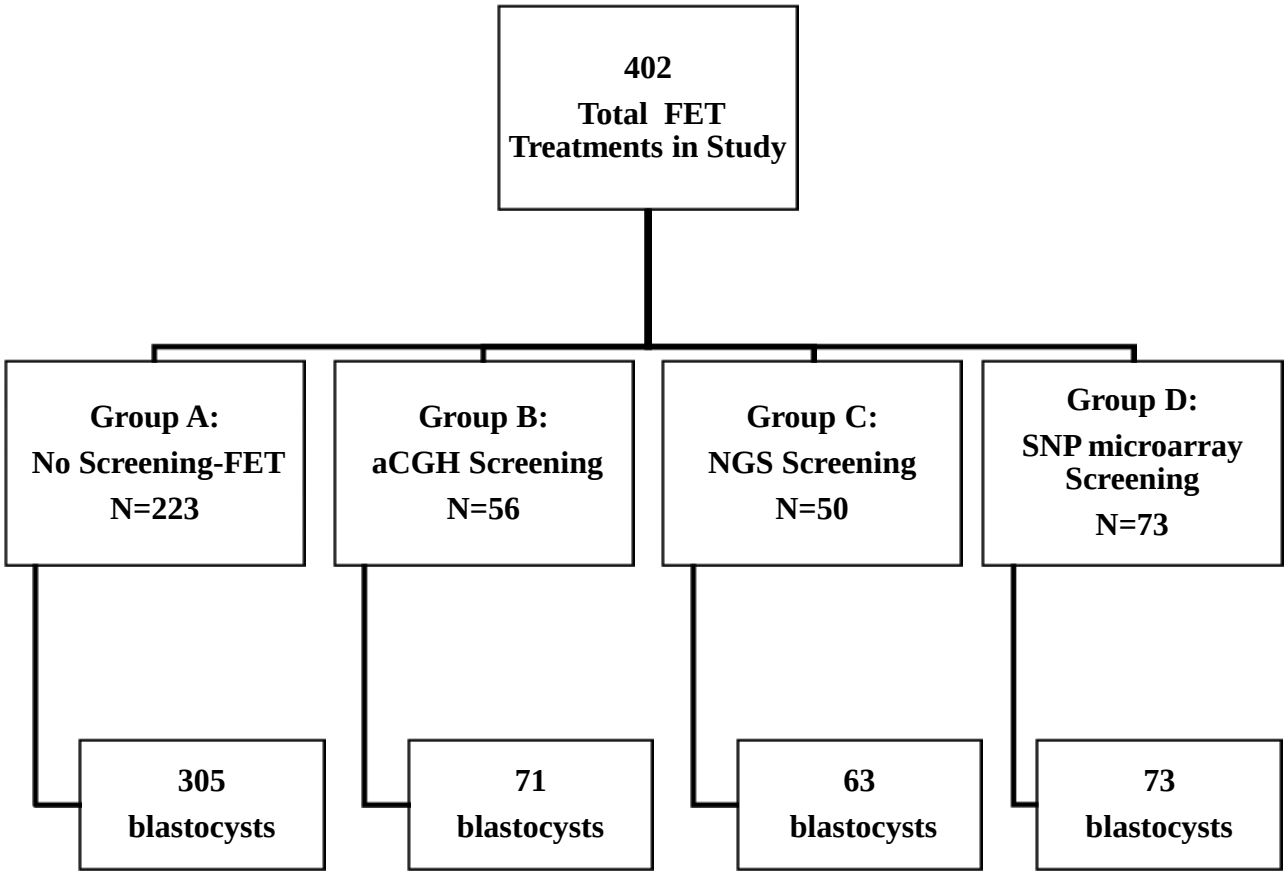



Figure 4.1. Grouping by screening method. Schematic for Frozen Embryo Transfer (FET) treatments included in each of the four groups.

4.2.3.1 Inclusion Criteria

Criteria for including embryos in this study are as follows:

- Observations for individuals in a non-repeated case study only, in which individuals who attempted their first uterine embryo transfer in a frozen embryo transfer (FET).
- All FETs, in which no trophectoderm biopsy was performed on the embryo during a fresh IVF treatment, where the embryo was cryopreserved at the blastocyst stage, were included in the reference group.
- All embryos that underwent trophectoderm biopsy procedures were included in groups B, C and D depending on the PGS technique used.

4.2.3.2 Exclusion Criteria

Criteria for excluding embryos from this study are as follows:

- Individuals who used donated gametes (oocytes or sperm cells).
- Individuals that did not have any embryos to cryopreserve or received at least one embryo transferred in the uterus during the fresh IVF treatment.
- Secondary FET treatments following a failed primary FET treatment.
- Embryos that were originally cryopreserved in fresh treatments and were warmed, biopsied and re-vitrified with the intension of re-warming only euploid embryo(s) for transfer in a subsequent FET.
- Adopted embryos that would represent the primary FET for a recipient
- Embryos that were not cryopreserved by the Center.
- Individuals who utilized more than one technique in an IVF treatment.

### 4.2.4 Embryo Culture and Trophectoderm Biopsy

Embryos were cultured in sequential media (VitroLife, G Series) for a period of 6 days in incubators set at 6.2% $CO_2$ and 5% $O_2$ (Sanyo MCO-5M). Upon evaluation of morphokinetic parameters of blastocyst development, the embryos were selected for trophectoderm biopsy. Each embryo was placed in a culture dish (Falcon 353655) with 5 µl drops of modified human tubal fluid (mHTF) (Irvine Scientific, SWM 9983-100) overlaid with 4 ml of culture oil (VitroLife, 10029). The embryos were placed individually in drops and the dish was placed on a heated stage (TokaiHit, heated plate) with temperature maintained at 37˚C. Biopsies were performed on an inverted microscope system (Olympus IX70) by ablation of trophectoderm cells using a Saturn Laser 40X objective. The Saturn Laser Lens consists of 1480 nm / 400 mW solid state diode laser with a pulse length range 0.005-2.0 ms / 5-2000 µs. A total of 4-6 cells were removed from the embryo and the cells were washed through 4, 20µl drops of wash buffer provided by each of the genetic testing centers. Each biopsy was loaded into a DNA tube labeled with the embryo number.

The tubes were held at -30˚C for a minimum of 2 h and then packaged and shipped with dry ice overnight to the reference genetic laboratory.

Amplification and interpretation of DNA from trophectoderm cells biopsied by the Center was performed by a reference laboratory. One of three techniques for the screening of biopsied cells to establish ploidy was utilized by the Center. Upon recipient of PGS results from the reference laboratory, warming of euploid single or double embryo transfer procedure was planned.

### 4.2.5 Blastocyst Vitrification

Immediately following the trophectoderm biopsy, blastocysts were individually vitrified (VitroLife, 10119). Each blastocyst was placed in Vitri 1 solution of the vitrification kit for 5 min for equilibration. The blastocyst was then placed in Vitri 2 solution of the kit for 2 min to initiate the cryoprotectants exchange. Finally, the blastocyst was placed in Vitri 3 solution for 45 sec to complete the vitrification process, in which the cells are dehydrated and the water volume within the cells is displaced by the cryoprotectants in the vitrification medium. This latter solution is viscous, which when frozen, results in extreme hardening to achieve a glass like state (Lowe et al. 2015). Each blastocyst was loaded onto the Rapid-I (VitroLife, 14406) device and stored in liquid nitrogen until scheduled warming.

### 4.2.6 Selection of Blastocysts for Transfer

Embryo selection for the reference group was made solely based on morphokinetic parameters. For the groups with PGS screening (B, C and D) selection was performed using a two tier method: euploid status followed by morphokinetics. In the screening groups, the highest morphologically graded one to two embryos were selected for warming on the morning of the

procedure from the embryos that were reported euploid through pre-implantation genetic screening (PGS). Embryos were graded using the Society of American Reproductive Technology (SART) guidelines (Racowsky et al. 2010).

### 4.2.7 Blastocyst Warming and Uterine Transfer

Blastocysts were warmed using the Rapid Warm (VitroLife, 10120) kit, consisting of proprietary solutions. The Rapid I straw was removed from liquid nitrogen and submerged in Solution 1 of the Rapid Warm kit for 2 min. The embryo was then moved to Solution 2 for 3 min followed by 5 min in Solution 3. Finally the embryo was moved into a pre-equilibrated culture dish containing G2 Plus (VitroLife, 10132) supplemented with 20% human serum albumin (VitroLife, 10064). The equilibration process involved the exposure of the culture dish overnight in an undisturbed cell culture incubator (Sanyo MCO-5M) programmed for 6.2 % carbon dioxide and 5% oxygen. This setting allowed for the bicarbonate buffered culture medium to equilibrate to a pH of 7.27 ± 0.07. Warmed embryos were cultured for a period of 2 h followed by evaluation of morphology. Embryos were then placed in the center well of the transfer dish (Falcon 35307). The blastocysts at uterine embryo transfer was placed in the transfer catheter by myself and passed to a physician. The physician guided the catheter in the uterus, at which point I expelled the contents of the catheter, blastocyst(s) in medium, in the uterine cavity.

### 4.2.8 Terminology

*Single embryo transfer (SET)* is defined as one blastocyst transferred to the uterus of the mother. *Double embryo transfer (DET)* is defined as two blastocysts simultaneously transferred to the mother's uterus. *Clinical pregnancy* is defined as two positive serum beta human chorionic

gonadotropin (bHCG) values on 10 and 12 days post uterine embryo transfer (ET). With two established quantitative values, which arise from day 10 to day 12, the individual is considered to have positive chemical pregnancy outcome. *Biochemical pregnancy* is defined as an initial positive quantitative value on 10 days post ET and a subsequent decline of the value on day 12 post ET. *Fetal Heart Beat (FHB)* is defined as presence of fetal heart beat revealed by ultrasound at 5 weeks post uterine embryo transfer. *Implantation rate* is defined as the number of embryos with measurable FHB observed per blastocyst transferred in that category. *Ongoing pregnancy rate* is defined by the number of individuals that have at least one FHB observed per IVF treatment in that category. *Miscarriage* is defined by at least one FHB observed following a subsequent loss of pregnancy prior to 20 weeks of gestation.

### 4.2.9 Statistical Methods

The distinct interaction of variables and their influence on IVF treatment outcome was found through the use generalized additive model (GAM) using linear regression and a permutation analysis with R software as part of the regression tree computational analysis (Huimin Liu 2008; Kuhn and Johnson 2013). Final fitting of the model was determined with a random forest algorithm (see Chapter 2), a method which determines classification of variables is using a regression approach. Decision tree construction was performed using the evolutionary tree algorithm using R software to maximize discrimination between groups at each node of the decision tree with maximal homogeneity in each split rule resulting from subset of the predictors (Grubinger et al. 2014). The evolutionary tree utilized regression and coupled it with a Bayesian information criterion (Fan and Gray 2005).

## 4.3 Results

### 4.3.1 Outcome Characteristics by Group

Primary evaluation of results by group yielded the highest clinical pregnancy rate success in group B (aCCH) for single embryo transfers (68.3%, n=28) and double embryo transfer (86.7%, n=13) categories (table 4.2). Overall ongoing pregnancy rate was also the highest in group B (66.1%, n=37). The overall miscarriage rate was lowest, again, in group B (9.8%, n=4). The overall chemical pregnancy and implantation rate were the highest in group B (aCGH) per embryo transferred (figure 4.2). The mean female age in the reference group consisted of a younger patient population (group A was 32.4 ± 3.7 years, B was 35.5 ± 4.1 years, group C was 35.1 ± 4.4 years and group D was 36.4 ± 3.5 years).

Table 4.2. Comparison of pregnancy and implantation outcomes within groups

| Parameter | Group A No PGS | Group B aCGH | Group C NGS | Group D SNP array |
|---|---|---|---|---|
| # of treatments with SET | 142 | 41 | 37 | 49 |
| # of treatments with DET | 81 | 15 | 13 | 24 |
| Clinical pregnancy rate with SET % ( | 59.8% (85) | 68.3% (28) | 51.4% (19) | 67.3% (33) |
| Clinical pregnancy rate with DET % ( | 66.7% (54) | 86.7% (13) | 53.8% (7) | 70.8% (17) |
| Overall clinical pregnancy rate % (n) | 62.3% (139) | 73.2% (41) | 52.0% (26) | 68.5% (50) |
| Overall implantation rate % (n) | 49.5% (151) | 63.4% (45) | 50.8% (32) | 56.7% (55) |
| Overall ongoing pregnancy rate % (n) | 55.6 % (124 | 66.1% (37) | 46.0% (23) | 60.3% (44) |
| Overall miscarriage rate % (n) | 10.7% (15) | 9.8% (4) | 11.5% (3) | 12% (6) |

*SET* single embryo transfer, *DET* double embryo transfer

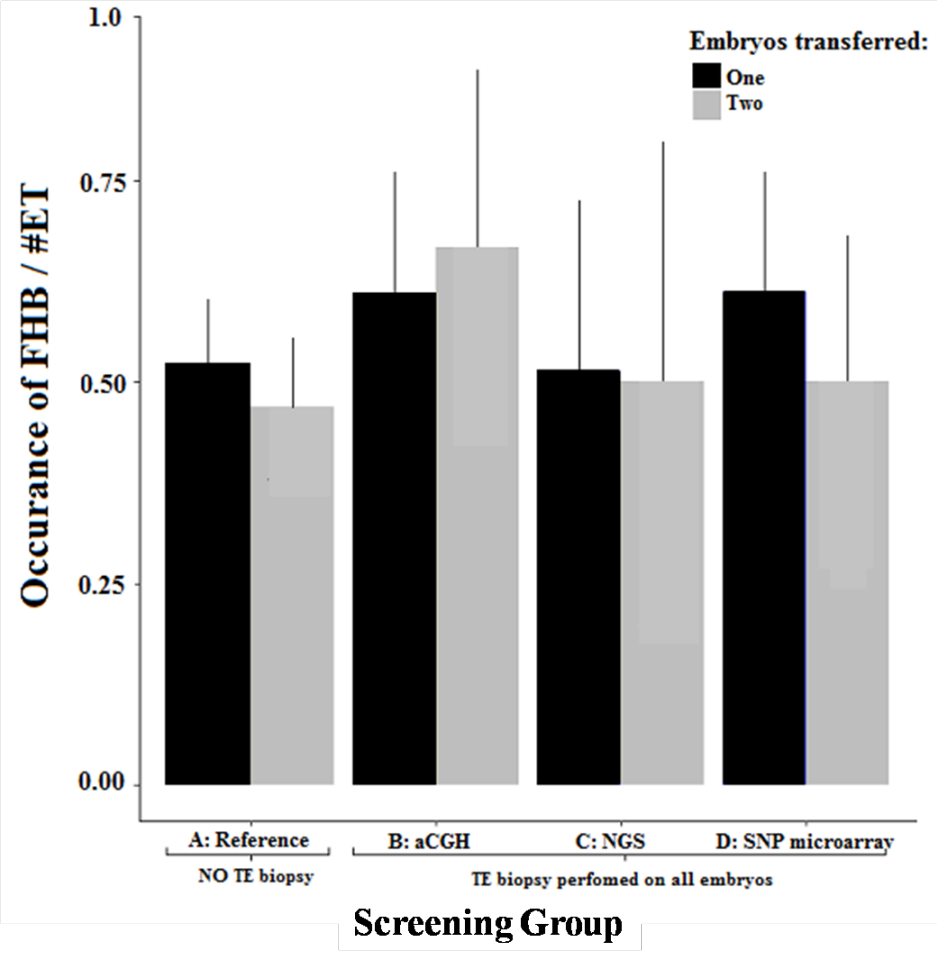



Figure 4.2. Success rate measured in presence of heartbeat. Comparison of mean occurrence of heartbeats ± standard error (SE), detected across all groups by number of embryos transferred.

### 4.3.2 Importance of Female Age

Permutation analysis of variables using Random Forest analysis (see Chapter 2) (Breiman 2001) determined female age as the variable, with the largest contributions to outcome ($P$<0.01, Gini index=74.28). FHB observed post-treatment ranked as the next most important ($P$=0.02, Gini index=17.01), followed by chemical pregnancy ($P$<0.01, Gini index=16.17) and finally number of embryos transferred ($P$<0.01, Gini index=13.59) (Table 4.3). The weighted importance assessment established that all four variables were interacting to influence outcome in the form of embryo viability, with respect to reference or screening group.

Table 4.3. Permutation of interactive variables

| Variables | Mean Decrease Gini Index* | *P* value** |
|---|---|---|
| Female age | 74.28 | 0.01 |
| Fetal Heart Beats (FHB) | 17.01 | 0.02 |
| Chemical pregnancy | 16.17 | 0.01 |
| Number of embryos transferred | 13.59 | 0.01 |

*_P values_ determined using Random Forest model.

**Mean Decrease Gini Index is a measure of impurity in the node. A large value is representative of greater role of variable in partitioning data into defined classes.

A decision tree was then formulated using these variables explaining the homogeneity in the data and describing the probabilities of IVF treatment outcomes (Figure 4.3). The first clear discrimination in the dataset was observed for individuals <34 years of age (node1). This age group had characteristics, which appear in the reference group with an average predictive probability of approximately 65%. Individuals in this age group did not benefit from the pre-implantation screening (n=186).

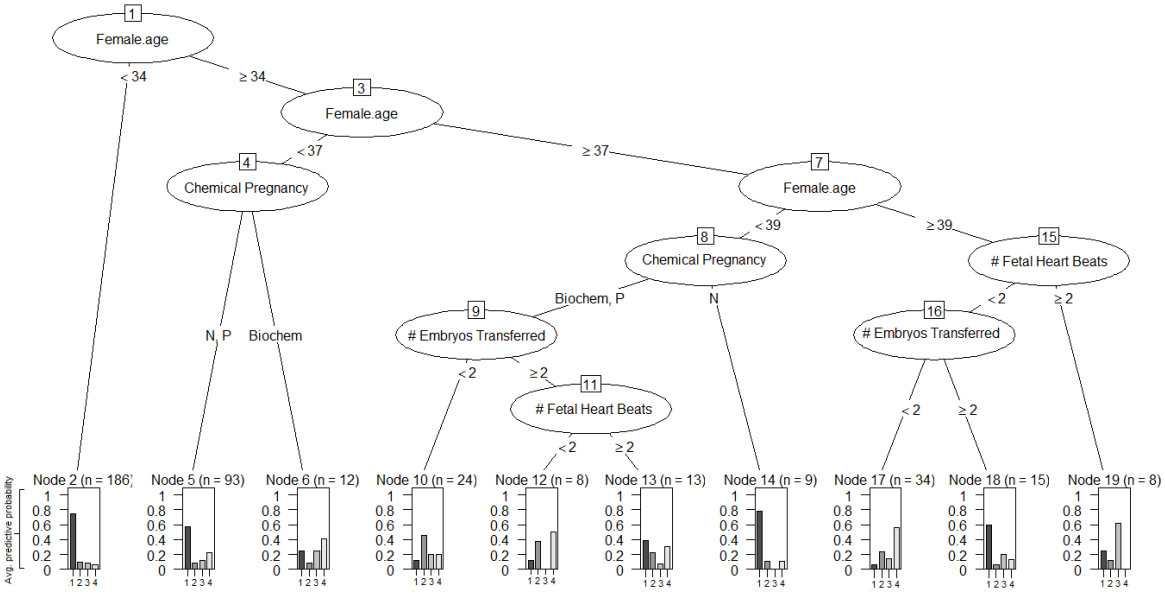



Figure 4.3. Tree for screening effect prediction. The terminal variables, determined by random forest, presented as graphs for predictive probability of an individual in that specific path of originating from one of the four groups. The variables of importance in creation of split rules are: age of female patient or oocyte source (Female age), number of embryos transferred , outcome expected as chemical pregnancy (Chemical Pregnancy) , and occurrence of beating fetal hearts observed (# Fetal Heart Beats).

Individuals 34-36 years of age had the highest average probability of having characteristics from the reference group based on a chemical pregnancy outcome of ~58% (nodes 4 and 5). Individuals in this class demonstrated a slight and equal benefit to any form of PGS technique with no differences observed among PGS techniques (aCGH, NGS or SNP array). Individuals of 37-38 years, benefited from any PGS techniques equally, without which pregnancy success was <20% (node 14, n=9). Single embryo transfer showed the highest rate of success with the least number of embryos transferred, resulting from aCGH screened euploid embryos with an average predictive probability of ~50% (n=24, node 10). Individuals >39 years benefited from PGS (nodes 17, 18 and 19). The highest success of this group was achieved when euploid embryos were transferred using NGS (node 19) with the average predictive probability of ~65% (n=8).

### 4.3.3 Accuracy Determination of Random Forest Analysis for Screening Effect

PGS groups were compared against the reference group to determine the accuracy and effect size of any final node on the random forest analysis. All terminal nodes in figure 4.3 predict decision results for all 4 groups. Therefore, the receiver operating curve analysis was performed for each of the PGS groups against the reference group. Any final node predicting that a PGS technique will yield in higher success than reference group has a higher success than chance alone (figure 4.4).

- Group B (aCGH) has an area under the curve (AUC) of 0.70 indicating that any final node of the decision tree will result in 14% greater success than chance alone as the lower measure of success and 27% greater success than chance alone as its highest measure of success determined by 95% confidence intervals (0.64 – 0.77).
- Group C (NGS) has an area under the curve (AUC) of 0.63 indicating that any final

node of the decision tree will result in 13% greater success than chance alone as the lower measure of success and 20% greater success than chance alone as its highest measure of success determined by 95% confidence intervals (0.57 – 0.70).

- Group D (SNP array) has an area under the curve (AUC) of 0.69 indicating that any final node of the decision tree will result in 14% greater success than chance alone as the lower measure of success and 26% greater success than chance alone as its highest measure of success determined by 95% confidence intervals (0.57 – 0.70).

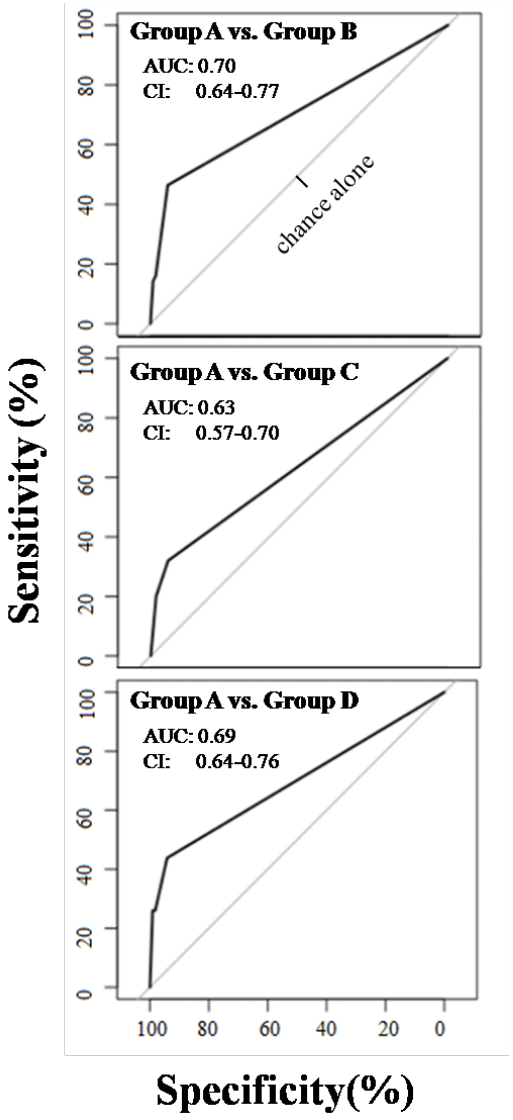



Figure 4.4. ROC analyses for screening effect. True positive rate (sensitivity) is plotted against the false positive rate (1-specificity). Each of the screening groups was compared against the reference groups for the area under the curve (AUC) using ROC analysis. Confidence intervals (95%) were determined using bootstrap CI to determine the effect size that takes specificity and sensitivity jointly into account demonstrating that the prediction model will results are higher than chance for each screening compared to the reference.

## 4.4 Discussion

Each screening group was expected to reduce the occurrence of miscarriages or spontaneous abortions with the use of euploid embryos (Handyside 2013; Gleicher and Orvieto 2017; Dahdouh et al. 2015). The results of the present study differ from previous claims that the utilization of PGS improves IVF outcomes while lowering miscarriages (Keltz et al. 2013). In the present study, no significant

improvement was observed in the reduction of miscarriages compared to the reference group, a finding in agreement with other studies in the literature (Gleicher et al. 2014; Gleicher et al. 2017). Although aneuploidy of whole chromosomes is prevalent and a leading cause of spontaneous abortions, it may be inaccurate to report segmental deletions or duplications of chromosomes in embryos as aneuploid. The evidence and understanding of embryology and mechanisms of self correction of DNA errors in early embryos is still not well understood.

Next generation sequencing has shed light on the importance of detecting aneuploidy at a level, which includes mosaicism as well as small segmental deletions or duplications of chromosomes in preimplantation embryos. This technology has proven to be most advantageous for women ≥39 years when rate of DNA copy error is the highest (figure 4.3, >39 year node). Yet, our hypothesis that accuracy of SNP arrays in determining euploidy of blastocysts yield the highest pregnancy outcome is rejected. Based on the data from the current study, a comparison of the three methods of PGS demonstrated NGS to be most efficient and beneficial in determination of embryonic ploidy and genomic errors specifically for individuals ≥37 years. Although SNP micro-array and aCGH are comparable for accuracy in terms of euploid embryos resulting in pregnancy, the rate of successful pregnancies decreases with advancing maternal age compared to NGS.

Based on the findings of this study, it is recommended that:

1) NGS be offered to individuals presenting with infertility of age ≥37 years
2) Younger individuals should be counseled and advised that NGS does not demonstrate statistically higher pregnancy success compared to aCGH and SNP microarray.

Mosaicism went unreported with both array Comparative Genomic Hybridization (aCGH) and single nucleotide polymorphism array (SNP array) techniques. As a result, greater than expected rate of biochemical pregnancies was observed for transferal of reported euploid embryos (which may have been mosaic). A higher rate of biochemical pregnancies is associated with the

transfer of a mosaic embryo when compared to the transfer of embryos that were euploid (Lledó et al. 2017; Daughtry and Chavez 2016). 1-2% of all preimplantation embryos across all maternal ages demonstrate mosaicism (Capalbo et al. 2017). As a result, since a minority of embryos are affected by mosaicism (<3%), NGS and technologies that are capable of detecting mosaic embryos will result in marginal improvement in clinical pregnancy outcome. Capalbo et al. (2017) failed to distinguish the variability in the rate of mosaic embryos due to maternal age. As demonstrated by the current study, NGS is most beneficial when maternal age is >39 years. This evidence suggests that mosaic embryos may be more prevalent in the older maternal population resulting in higher rates of spontaneous abortions. Identification of segmental chromosomal mosaicism and partial chromosomal mosaic cells lines in these embryos is now possible due to NGS technology, making it an asset in embryo selection for pregnancy with IVF.

In direct contradiction to the study of Capalbo et al. (2017), which showed 1-2% prevalence of mosaicism in preimplantation embryos, greater mosaicism rate is observed in a significant proportion of biopsies analyzed by NGS (Munné and Wells 2017). Furthermore, Munné and Wells identify a positive correlation of maternal age and rate of embryonic mosaicism. Aneuploidy in embryos of advanced maternal age individuals as the product of non-disjunction during meiosis is more likely. Aneuploidy is also explained by partial monosomies or trisomies and segmental loss of chromosomal content. Munné and Wells (2017) confirmed an increase in miscarriage rate from mosaic embryos compared to pregnancies achieved with euploid embryo transfers. In the past, the use of aCGH yielded the highest success in pregnancy outcome when a euploid embryo was transplanted in individuals of <35 years, but was significantly less effective in the older maternal age groups. Single nucleotide polymorphism array demonstrated a higher rate of success compared to aCGH due to greater in depth sequencing method. SNP also demonstrated a decrease in rate of

success with advancing maternal age. NGS identifies errors beyond whole chromosome deletions or duplications and large segmental deletions or duplications, it identifies mosaicism, thereby, reducing the rate of spontaneous abortions and ultimately increasing the rate of sustainable pregnancy in individuals with advanced maternal age.

## 4.5 Conclusion

Evidence from secondary analysis with interactions from the current study has established that the use of PGS for individuals of $\leq$34 years of age has no benefit. Patients should be counseled appropriately to minimize unnecessary biopsy procedures on embryos and to minimize patient financial burden. Furthermore, the results of the current study demonstrate no significant difference in pregnancy outcome of individuals of maternal age between 35-37 years when compared across the PGS techniques (aCGH, SNP array and NGS). Individuals should be counseled appropriately and a decision, of which PGS techniques to utilize should include procedural costs. Other factors in patient diagnosis (i.e., DOR) should be reviewed to determine if PGS is beneficial. A final conclusion is that NGS should be used for individuals of advanced maternal age ($\geq$38 years) to maximize success outcome of this sub-population. Future studies, which include specific rate of mosaicism of embryos by maternal age and the error rate in misdiagnosis or false positive detection of mosaic lines in embryonic biopsied samples by maternal age are needed to understand the implications of NGS technique on clinical outcome.

# CHAPTER 5

# FOLLICLE STIMULATING HORMONE AND ANTIMÜLLERIAN HORMONE AS INDICATORS OF EMBRYONIC DEVELOPMENT

## 5.1 Introduction

The condition of diminished ovarian reserve (DOR) is characterized by poor fertility outcomes and currently presents as a major challenge in reproductive medicine, particularly in vitro fertilization (IVF) (Cohen et al. 2015; Atasever et al. 2016). Women are diagnosed with DOR if they demonstrate at least one of the following:

- high follicle stimulating hormone (FSH) levels when not in the follicular phase
- low antral follicle counts
- low anti-müllerian hormone (AMH)

A woman in her reproductive years has good prognosis when her FSH is between 3.1-7.9 mIU/ml and her AMH is ≥1 ng/ml (Atasever et al. 2016; Muasher et al. 1988; Abdalla 2004). AMH is used to assess the remaining ovarian reserve as well as predictor of response to controlled hyperstimulation of the ovaries during IVF treatment (Broer et al. 2011; Tal et al. 2015; de Vet et al. 2002).

Women with elevated basal FSH are reported to still achieve reasonable pregnancy rates, but few studies report correlation with blastocysts development (Safdarian et al. 2012; Broekmans et al. 2009; Levi et al. 2001; Esposito et al. 2002). The purpose of the current study is to understand the interaction between the number of mature follicles at trigger, AMH and basal FSH values with blastocysts development in DOR and POF patients as compared to good prognosis patients. The current study examines the correlation between diagnostic findings of basal FSH and AMH values and their predictive value in embryo development. Diagnostic values of AMH/basal FSH can be

resourceful in determining the optimal simulation protocol and identification of individuals who will not benefit from IVF due to poor prognosis. I hypothesize that individuals who have an AMH of ≤1 ng/ml and a basal FSH greater than 8 mIU/ml have significantly lower percentage of high quality embryos compared to patients that have values considered normal for fertility.

## 5.2 Material and Methods

This study is prospective (data collected forward in time) analysis of frozen embryo transfer treatments performed at a single site, Dallas IVF, referred to as "the Center[6]." The observational data included IVF treatments over a six month period (January 1, 2015 to June 31, 2015). The data for treatments included in this study were retrieved using an electronic database management system (eIVF, USA), in which the data was de-identified to remain in compliance with Health Information Portability and Accountability Act (HIPAA).

### 5.2.1 Study Population

All patients that underwent IVF treatment were included in the study regardless of age or prognosis. The most recent basal FSH level (samples drawn on days 2-4 of menses) was obtained prior to initiation of treatment. Patients were treated with either of the following protocols:

- GnRH (Gonadotropin releasing hormone ) antagonist: This protocol includes the use of medication such as Ganarelix for the suppression of luteinizing hormone (LH) surge and ovulation by blocking the secretion pituitary gonadotropins, supplemented by medication containing follicle stimulating hormone (FSH) to stimulate follicular growth and development and

[6] *Dallas IVF* is a registered member of the American Society of Reproductive Medicine and a Society of Assisted Reproductive Technology. The facility is accredited by College of American Pathology (CAP), Clinical Laboratory Improvement Amendments (CLIA) and is registered with the Food and Drug Administration (FDA).

a medication containing human chorionic gonadotropin to induce final maturation of oocytes within follicles prior to ovarian retrieval (Hebisha et al.2017; Lai et al. 2013).

- Long agonist protocol: This protocol includes the use of a pituitary down-regulation during the luteal peak period (7-10 days prior to the start of the menstrual cycle) to prevent premature luteinization and ovulation (Siristatidis et al. 2015). After this initial period, medications containing FSH are added to promote follicular development. Finally HCG medication is administered for final oocyte maturity prior to retrieval (Lai et al. 2013; Surrey 2007).

All patients who underwent ovarian stimulation and subsequent IVF culture were included in the study.

### 5.2.2 Hormone Assays and Follicular Measurements

Endocrine assays for serum FSH, AMH and estradiol concentrations were performed by a reference laboratory (LapCorp) using liquid chromatography/tandem mass spectrometry (LC/MS-MS) (Ketha et al. 2014; Koal et al. 2012). Measurements of follicular development were performed at the Center by a sonographer certified by American Registry for Diagnostic Medical Sonography (ARDMS). Follicle growth was measured in millimeters and was a mean diameter per follicle. Calculations of the mean were provided by the ultrasound software.

### 5.2.3 Treatment Protocol

Controlled ovarian stimulation was carried out for all patients using either the down regulated agonist long protocol or the antagonist protocols, as previously described. A transvaginal scan was performed prior to stimulation to ensure ovaries were free of cysts. Both protocols incorporated either recombinant FSH and hMG (human menopausal gonadotropins) or urinary

FSH. For the long protocol, down regulation of patients was obtained through leuprolide (Lupron®) administration beginning mid-luteal phase. For the antagonist protocol GnRH (gonadotropin releasing hormone) was administered when the lead follicle reached 15mm in diameter or the latest by day 7 of stimulation. When a majority of the follicles developed between 16-24mm in diameter and serum estradiol levels were rising appropriately to reflect oocyte maturation, the patients were then triggered for oocyte retrieval by the administration of 10,000 IU of human chorionic gonadotropin (HCG) for either protocol or Lupron trigger with the antagonist protocol. Oocyte aspiration was performed under ultrasound guidance between 36-38h post trigger administration. Insemination of oocytes and culture of embryos was performed over a six day period following transvaginal oocyte retrieval (TVOR). Embryos were graded using the SART grading system (Heitmann et al. 2013).

### 5.2.4 Definition of Outcome

The following definitions apply to the outcomes measured in this study.

- *Minimum AMH* is defined as the lowest measured anti-müllerian hormone level (ng/ml) prior to the start of IVF treatment.
- *Maximum FSH* is defined as the highest follicle stimulating hormone level (mIU/ml) measured on menstrual IVF treatment day 3.
- *Mature follicle* is defined as a follicle which measures $\geq$16mm in diameter.
- *Estradiol (E2) at trigger* is defined as the highest measured serum estradiol level (pg/ml) prior to trigger medication administration.

- *Total IU medication* is defined as the total international units of gonadotropins (FSH/LH) administered over the length of the follicular stimulation IVF treatment. *Day of trigger* is defined as the stimulation day upon which either HCG or Lupron® were administered.

- *High quality blastocysts* are defined as the number of blastocysts that were usable for cryopreservation or uterine transfer.

- *Success* is defined as >40% of fertilized oocytes developed into high quality blastocysts per IVF treatment.

### 5.2.5 Data Analysis

The distinct interaction of variables and their influence on IVF treatment outcome was analyzed through the use of a generalized linear model (GLM) employing logistic regression and survival analysis with R software (Huimin Liu 2008; Kuhn and Johnson 2013). Final fitting of the model was determined through the use of random forest algorithm, a method which determines classification of variables using a regression approach. Decision tree construction was performed using the evolutionary tree algorithm using R software to maximize discrimination between groups at each node of the decision tree with maximal homogeneity in each split rule resulting from subset of the predictors (Grubinger et al. 2014). The evolutionary tree utilized regression and coupled it with a Bayesian information criterion (Fan and Gray 2005).

## 5.3 Results

Data evaluated for individuals who produced >12 mature follicles was compared to individuals who produced $\leq$12 mature follicles at trigger. The latter group consists of individuals presenting with diminished ovarian reserve or preliminary ovarian failure. Total IUs of medication

were approximately 1000 international units (IU) more per IVF treatment compared to the group producing more mature follicles (Table 5.1). Despite the larger quantity of drugs, the estradiol at trigger were observed to be approximately 1700 pg/ml lower than individuals who produced >12 mature follicles at trigger.

The generalized linear model determined minimum AMH, total IU medication, number of mature follicles at trigger and E2 at trigger as significant actionable contributors in optimizing blastocyst development per IVF treatment. A median percentage of 40% fertilized oocytes developed into high quality blastocysts was 40% (table 5.1). Evtree analysis was performed to optimize blastocyst development per IVF treatment to discriminate outcome over the median. The analysis demonstrated that individuals whose E2 at trigger was <1584 pg/ml had a 80% probability of ≥40% blastocyst development success (n=7, node 2) (Figure 5.1). When E2 at trigger was ≥1584 pg/ml, the number of mature follicles at trigger <12 and total IU medication <3025 resulted in 90% blastocyst development success (n=10, node 5). Total IU medication could be above 3025 IU for this group if E2 at trigger was <2748 pg/ml and minimum AMH was <1.783 ng/ml for 80% blastocyst development success (n=8, node 11). Higher minimum AMH and E2 at trigger for this group resulted in <15% success (nodes 9, 10). When E2 at trigger was >1584 pg/ml and ≥12 mature follicles were present, success was improved by administering <3025 total IU medication with trigger administered by the 10th day of stimulation (node 13). If the E2 at trigger was <3056 pg/ml, 70% success was observed (n=7, node 14). E2 at trigger could exceed 3056 pg/ml only if maximum FSH was ≥6.5 mIU/ml resulting in 85% success (n=12, node 17).

Table 5.1. Comparison of treatments reflected by number of mature follicles at trigger

| Parameter | > 12 mature follicles n=61 | ≤12 mature follicles n=45 |
|---|---|---|
| Female age (years) | 34.4 ± 0.6 | 35.9 ± 0.7 |
| Male partner age (years) | 37.9 ± 0.9 | 38.6 ± 0.9 |
| Total IUs of gonadotropins/IVF treatment | 3070.1 ± 191.0 | 4110.8 ± 138.6 |
| Estradiol level at trigger (pg/ml) | 4547.8 ± 257.6 | 2821.6 ± 227.9 |
| Max basal FSH (mIU/ml) | 6.6 ± 0.4 | 7.8 ± 0.2 |
| Min AMH (ng/ml) | 5.4 ± 0.5 | 3.2 ± 0.7 |
| # Oocytes retrieved | 22.9 ± 1.3 | 14.9 ± 1.4 |
| # Oocytes normally fertilized | 14.2 ± 0.9 | 8.8 ± 1.0 |
| # High quality blasts | 5.4 ± 0.4 | 3.3 ± 0.4 |
| %High quality blasts | 38.9 ± 3.5 | 38.9 ± 2.4 |

*Mean values are reported ± std. error*

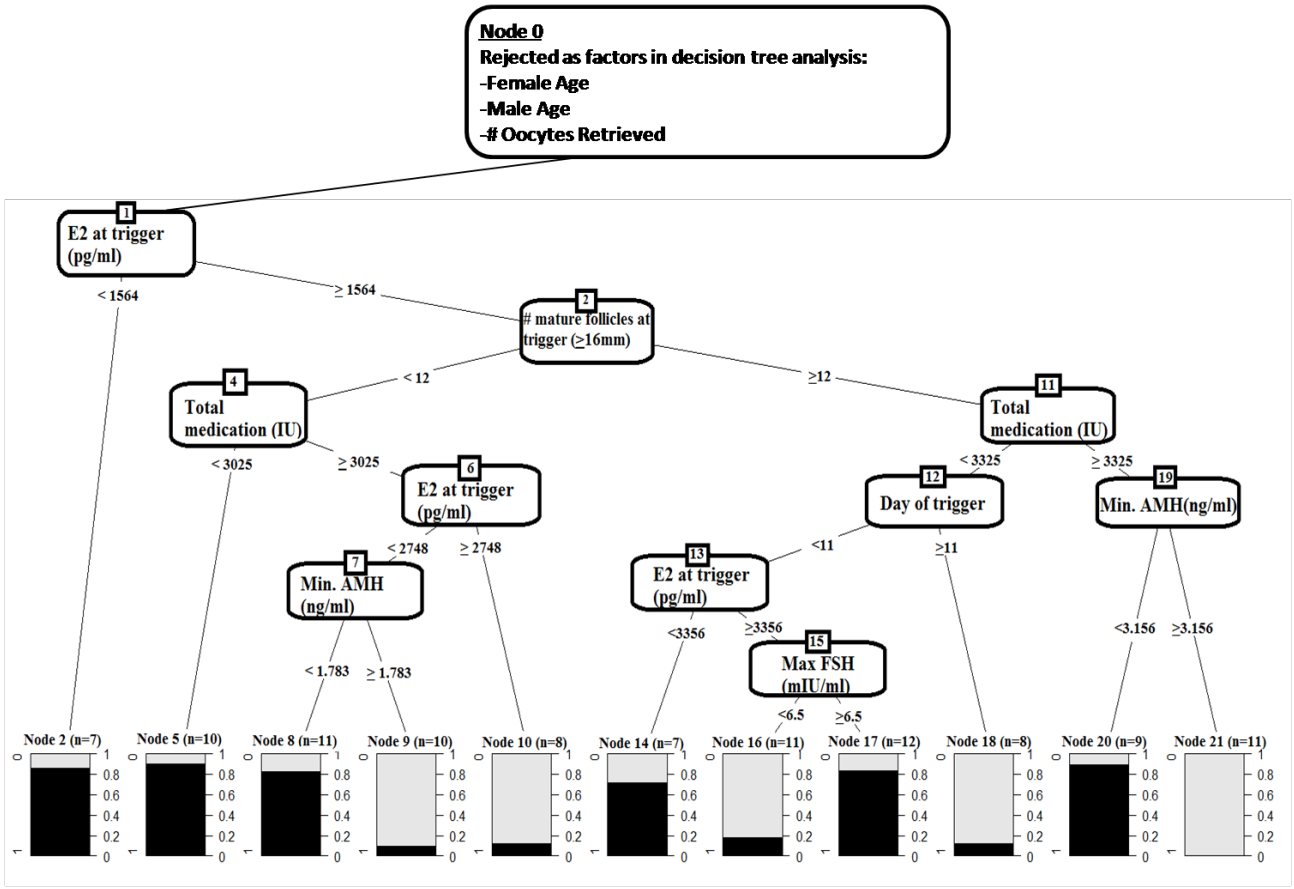



Figure 5.1. Tree for blastocyst optimization. Final graphed nodes depict successful treatments (≥40% blastocyst development, dark shading). Failed treatments (<40% blastocyst development) are shaded light. Values on the right of each bar represent the percent of successful treatments.

Success was over 90% when E2 at trigger was ≥1564pg/ml, ≥12 mature follicles at trigger, >3325 IU medication and minimum AMH of <3.156 ng/ml (n=9, node 20). The accuracy of the decision tree was calculated at 86.5 percent with an error rate of 13.5%. The receiver operating characteristic (ROC) curve area is roughly .87 (Figure 5.2) (see Chapter 2) (Robin et al. 2011) . Confidence intervals of 0.7996 - 0.9312 indicate that any final node on the model will yield 29% greater success than chance alone at the lowest predictive accuracy and 43% greater success than chance alone at its highest predictive accuracy.

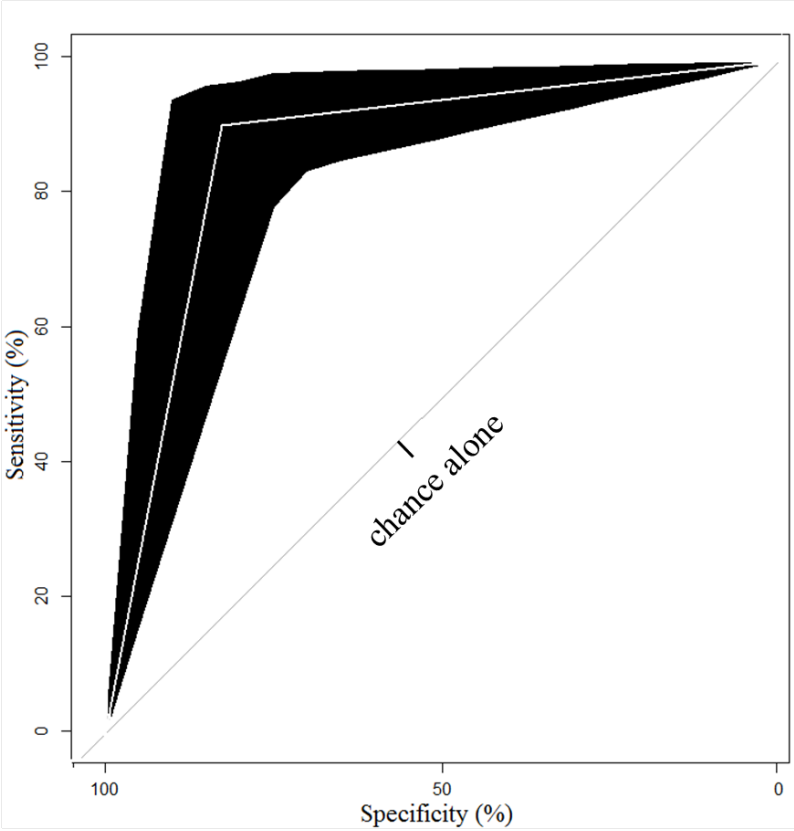



Figure 5.2. Receiver operating characteristic curve: blastocyst optimization model. True positive rate (sensitivity) is plotted against the false positive rate (1-specificity). The dark areas are the 95% confidence intervals.

## 5.4 Discussion

Basal FSH level and minimum AMH levels are routinely used as indicators of patient's pregnancy outcome or indication of IVF treatment prognosis. Yet, there remains a gap in

understanding how these values correlate with blastocyst developmental potential. Elevated basal FSH levels have been associated with a reduced overall live birth rate compared to individuals with normal levels (Abdalla and Thum 2004; Butts et al. 2013). I suggest that the live birth rate can be improved if the overall percent blastocyst production per IVF treatment is optimized by utilizing the decision tree of the current study. Surprisingly, individuals with good ovarian reserve (AMH >3.2 ng/ml) have an overall decreased blastocyst development rate compared to individuals with lower reserve. Larger numbers of follicles developing simultaneously may demonstrate slowed growth on ovarian scans since the gonadotropin dose is shared between follicles. As a result, gonadotropin doses are increased by clinicians to promote growth, which substantially relegates overall blastocyst development. Individuals with a poor prognosis due to diminished ovarian reserve (DOR) or preliminary ovarian failure (POF) shown here to have <12 mature follicles at trigger (Figure 5.2, node 3) demonstrate greater success when gonadotropins are <3025 total IUs and estradiol at trigger is under 2750 pg/ml. The correlation between the rate of follicle development and total IU of gonadotropins administered during stimulation produced a lowered rate of blastocyst developmental success when the patient was over-exposed to gonadotropins (Figure 5.1, node 21), as demonstrated by 0/11 successful treatments in this study.

I reject the hypothesis that patients who have an AMH of $\leq$1 ng/ml and a basal FSH greater than 8 mIU/ml have significantly lower percentage of high quality embryos compared to patients that have values considered normal for fertility. Instead, the current study indicates that individuals with elevated FSH had $\geq$12 mature follicles at trigger and over 80% of treatments were successful when total medication doses are <3325 IU. Thus elevated FSH levels do not indicate poor ovarian reserve or lower success (Table 5.1). I found that AMH values are reliable indicators of blastocyst developmental potential compared to basal FSH values. Patients presenting with decreased AMH

levels generally have <12 mature follicles at trigger and over 80% of treatments were successful when estradiol at trigger was less than 2750 pg/ml even when total IU of medication exceeded 3000 IU. I confirmed that patients with DOR, POF or poor prognosis overall exhibit overall a high rate of good quality blastocyst development per IVF treatment, which positively correlates with increasing levels of gonadotropins contrary to individuals with a higher number of mature follicles at trigger.

## 5.5 Conclusion

IVF treatments for patients that present with infertility due to DOR or POF, if managed appropriately, can produce higher rates of usable blastocyst production per IVF treatment, thus increasing rate of blastocyst developmental success. A greater number of high quality blastocysts increase the rate of pregnancy outcome per IVF treatment. This results in lower costs of treatments for the patient and lower emotional burden. Future studies are recommended with larger IVF treatment numbers that are fit to our decision tree for determination of applicability of the tree to new treatments.

# CHAPTER 6

# EFFECTS OF CULTURE pH AND PARTIAL PRESSURES OF OXYGEN AND CARBON DIOXIDE ON BLASTOCYST DEVELOPMENT

## 6.1 Introduction

One of the primary responsibilities of embryologists who perform human *in vitro* fertilization is optimization of embryo culture conditions to provide optimal embryonic development (and ultimately maximization of live births). Although pregnancy and live birth are the end goal of *in* vitro fertilization, our primary concern and focus was on the percent of high quality blastocyst production per treatment for optimization of all aspects (i.e., lessen patient financial burden, maximize high quality blastocyst development per treatment to satisfy patients' family building needs) associated with IVF especially for patients with diminished ovarian reserve or preliminary ovarian failure.

The primary environment of developing embryos *in vitro* consists of culture medium and its physio-chemical characteristics regulated by incubation. Culture conditions greatly influence embryonic cellular metabolism as well as gene expression in different species of mammalian embryos (Bauer et al. 2010; Gardner et al. 2013; Giritharan et al. 2012; de Los Santos et al. 2015). Optimal culture, in part, is a balance of pH and partial pressures of carbon dioxide ($pCO_2$) and oxygen ($pO_2$). The pH experienced by the embryos is a measure of hydrogen ion concentration, formed from the reaction between sodium bicarbonate in the medium and $CO_2$ gas in the incubator chamber. The pH of the bicarbonate buffed medium rises as $pCO_2$ in the incubator decreases. Maintenance of medium pH within acceptable ranges is critical in providing optimal medium nutrient status such as essential amino acids, hyaluronan, proteins and lipoic acid (anti-oxidant) found in the bicarbonate buffered culture medium (Gardner and Kelley 2017; Swain 2010; Sifer

et al. 2009). Variability in these conditions may lead to improper nutrient status in the medium, which negatively impacts metabolism of the embryo and its ability to express parts of the genome on time to maintain optimal development. Culture medium is held close to physiological conditions for pH and partial pressures of oxygen and carbon dioxide, with typical incubation levels being 6.2% ±0.3 $CO_2$ and 5.0% ±0.2 $O_2$. Normal ranges for pressure of dissolved gasses in IVF culture medium, when incubator settings are 6.2% $CO_2$ and 5.0% $O_2$, are as follows (according to the culture media manufacturer, iStat blood gas analyzer guide and the Center's protocol manual):

- $pO_2$ 83-108mmH
- $pCO_2$ 32-40 mmHg
- pH 7.20 to 7.34

If pH measures within normal range, $pCO_2$ and $pO_2$ ranges may be expanded by 10% according to manufacturer's recommendations.

Intriguingly, a lower rate of high quality blastocysts is observed for embryos in culture from one patient compared to another using the same medium batch, despite this emphasis on constancy of conditions. This difference in overall rate of development may be due to subtle, yet important environmental conditions that can be explained and identified with quality assurance (QA) studies. Commercially produced culture medium is accompanied by certification of analysis (CoA) documenting that any given batch of media has undergone a robust QA study with the manufacturer. Additionally, prepared medium is often convenient for use and storage. Despite the CoA, it is critical to perform additional inter-laboratory QA assessment and quality control (QC) study to verify that the manufacturer's results are reproducible and results produced onsite are within the allowable ranges for pH, partial pressures of $pO_2$ and $pCO_2$. Additionally, blastocyst

formation rate per batch may also be analyzed to ensure quality within the IVF laboratory. This aids in a timely identification and, if needed, calibration of faulty equipment or internal adjustments of gas levels per incubator. Extensive recommendations are available on medium pH (Swain et al. 2016; Wale and Gardner 2016; Gardner and Kelley 2017).

Despite an industry focus on quality assurance, implications -if any- of small variations in level of $pCO_2$ and $pO_2$ in the medium to embryo development have not been established. Consequently, my study has analyzed how the rate of blastocyst development is influenced by variation in pH, $pCO_2$ and $pO_2$ during culture. The goal was to identify whether currently acceptable ranges of culture pH, $pCO_2$ and $pO_2$ need to be further narrowed to optimize IVF treatment outcome and/or if there is an "incubator effect" influencing the percentage of high quality blastocysts. The purpose of this study is to understand how embryonic development is influenced by culture conditions associated with subtle variations of pH and partial pressures of oxygen and carbon dioxide in the medium. I hypothesized that subtle variation in incubator conditions can lead to poor blastocyst development.

## 6.2 Material and Methods

### 6.2.1 Location and Data Management and Study Population

This study is a prospective analysis of IVF at a single site, Dallas IVF, referred to as "the Center[7]." The data for treatments included in this study were collected using an excel spreadsheet with manual data entry for culture conditions as well as electronic database management system (eIVF, USA). Data were de-identified to remain in compliance with Health Information Portability

[7] *Dallas IVF* is a registered member of the American Society of Reproductive Medicine and a Society of Assisted Reproductive Technology. The facility is accredited by College of American Pathology (CAP), Clinical Laboratory Improvement Amendments (CLIA) and is registered with the Food and Drug Administration (FDA).

and Accountability Act (HIPAA). All IVF treatments performed from November 1, 2016 to Feb 28, 2017 at the Center were included in the study with the exception of oocyte and zygote warming (previously vitrified) treatments. A total of 42 treatments included in the study were initially organized into two groups for primary analysis of treatment characteristics with <12 oocytes retrieved designated group A(1˚) (n=27) and ≥12 oocytes retrieved group B(1˚) (n=15) based on statistical analysis and findings from Hariton et al. (2017) and Zhou et al. (2017). Group A, included patients presenting with diminished ovarian reserve or preliminary ovarian failure where as group B, included all other patients. The groups were subsequently organized to examine treatment characteristics with <14 oocytes retrieved Group A(2˚) (n=32,) and ≥14 oocytes group B(2˚) (n=10,) as it was determined by the Evtree analysis as significant in outcome determination.

### 6.2.2 Laboratory Environment and Testing

Sanyo incubators (Model-MCO-5M) were measured daily for chamber gas levels to ensure proper calibration of set values (6.2±0.3% for $CO_2$ and 5.0±0.2% for $O_2$). Chamber gas was measured daily using a G100 gas analyzer, for which a calibration curve was validated (Chapter 3,) (Viasensor). Embryo culture dishes were prepared using 35mm Petri dishes (Thermo Scientific™ 153066). Seven- 40 µl drops of culture medium, G1™ Plus (VitroLife, 10128) for the first three days then G2™ Plus for days 3-6 (VitroLife, 10132) were placed in the dish and overlaid with 4ml of culture oil (VitroLife, 10029). The dishes were placed in the incubator for equilibration for a minimum of 12 hours (see Chapter 1). Each batch of culture medium was tested for pH, $pCO_2$ and $pO_2$ using the *i*STAT1 handheld (Abbott) post-equilibration. A sample of the equilibrated medium (0.1 ml) was drawn up from the culture dish via syringe and was injected into the *i*STAT cartridge for testing. Results for each batch in each incubator were collected once per day over a

period of three consecutive days. The mean values were calculated for pH, $pCO_2$ and $pO_2$ per incubator, per batch. Embryos were cultured in sequential medium (VitroLife, G Series $^{TM}$) in one of three designated incubators assigned based on incubator availability. Embryos were graded according to the SART grading system (Heitmann et al. 2013) using an inverted microscope (Olympus IX70).

### 6.2.3 Definition of Outcome and Terminology

*Good quality day 3* embryos were defined as embryos that have cleaved into 6-8 cells, had <10% cellular fragmentation and exhibit moderate to perfect symmetry according to SART grading criteria (see Chapter 1). *High quality blastocysts* were defined as embryos on days 5 and 6, which exhibited an expanded zona pellucida, a compacted inner cell mass and a symmetrical cell organization in the trophectoderm. *Success* was defined as $\geq$60% of fertilized oocytes developing into high quality blastocysts for the primary Evtree analysis and $\geq$65% fertilized oocytes developing to high quality day 3 embryos per treatment for the secondary Evtree analysis.

### 6.2.4 Data Analysis

Principal components analysis was performed with SigmaPlot software. All data were reported as mean ±1 standard error. Additionally, decision tree construction was performed using the evolutionary tree algorithm (Evtree) using R software (Grubinger et al. 2014; Fan and Gray 2005) (see Chapter 2). The receiver operating characteristic (ROC) curve analysis, which illustrates Evtree's diagnostic ability as the discrimination threshold is varied for the statistical computation (Robin et al. 2011), was performed. The ROC curve calculates the rate of sensitivity

given the optimal specificity. With both specificity and sensitivity computed, an overall accuracy of the model was calculated.

## 6.3 Results

### 6.3.1 Culture Medium Conditions

Daily chamber gases measurements had a mean deviation from set-point of 0.0 $\Delta CO_2$ for all 3 incubators, 0.0 $\Delta O_2$ for incubators #1 and #2 and 0.1 $\Delta O_2$ for incubator #3. Culture media conditions were measured by the i-Stat. Incubator #2 had within-range values for G1 Plus and G2 Plus for pH, $pCO_2$ and $O_2$. Both Incubators #1 and #3 had out-of-range measurements for both G1 Plus and G2 Plus (table 6.1). Despite this consistency in measured chamber gas concentrations, incubator #3 resulted in out-of-range values for pH, $pCO_2$ and $O_2$ for all batches of both sequential medium as well as pH and $pCO_2$ for the primary batch of G1 Plus (table 6.1). Additionally, $pCO_2$ in solutions (G1 Plus and G2 Plus) were measured out-of-range in incubators #1 and #3 but within range in incubator #2.

### 6.3.2 Characteristics of the Study Population

#### 6.3.2.1 Primary Grouping

Initial analysis of the data was performed by evaluating the data in two categories: Group A(1˚) (<12 oocytes, n= 27) and group B(1˚) (≥12 oocytes, n=15), in which group A(1˚) included DOR and POF patients who are expected to have fewer blastocysts compared to group B(1˚) due to fewer oocytes retrieved. As expected, the number of oocytes inseminated, number of oocytes normally fertilized, number of good quality embryos on day 3 of development and the number of high quality blastocysts on day 5 were all higher per treatment in group B(1˚) (figure 6.1).

Interestingly, mean number of normally fertilized oocytes in group A was 4.3 ±0.4, of which a mean number of 2.4 ±0.3 embryos developed into good quality blastocysts (56% success per treatment). This comprised a higher success than group B(1˚), which had 8.9 ±1.1 normally fertilized oocytes, of which only 4.3 ±1.1 embryos developed into high quality blastocysts (48% success per treatment), thus lower rate of high quality blastocysts per treatment despite greater number of oocytes initially.

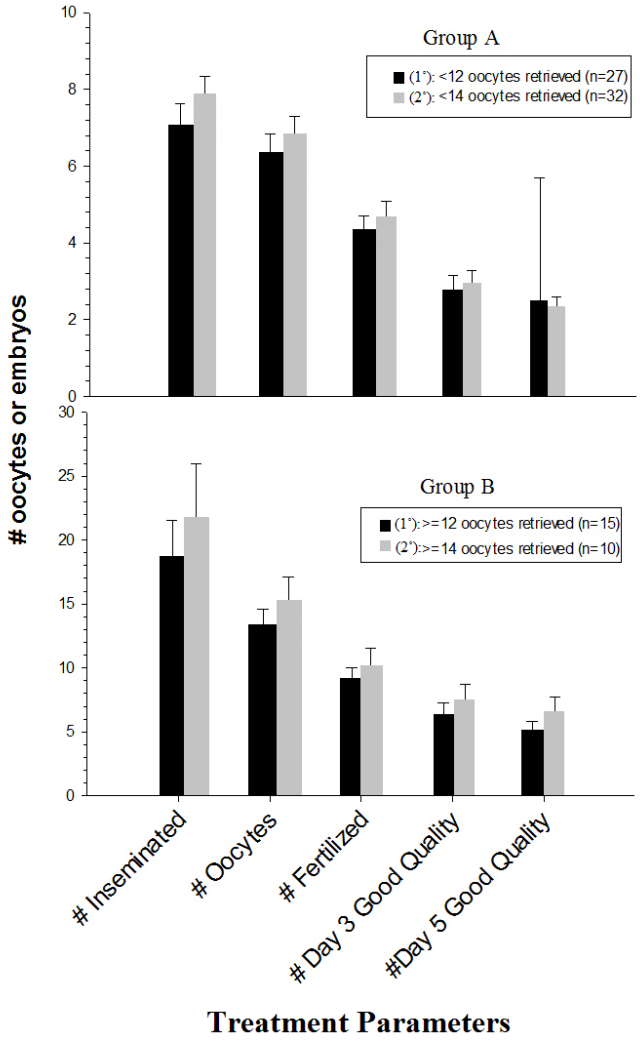



Figure 6.1. Treatment characteristics. Data demonstrate outcomes observed for patients in group A (1˚: <12 oocytes retrieved, 2˚: <14 oocytes retrieved) compared to group B(1˚: ≥12 oocytes retrieved, 2˚: ≥14oocytes retrieved.

6.3.2.2 Secondary Grouping

Decision tree results (discussed next) were conducted to understand the relationship between variations in population characteristics based on oocyte number. Based on this analysis, reorganization of the groups was conducted to include all treatments with <14 oocytes in group A(2˚) and ≥14 oocytes retrieved in group B(2˚). The number of high quality blastocysts in group A(2˚) was 2.9 ±0.3 (49%) compared to 6.4±1.3 (63%) in group B(2˚). These results demonstrated an expected rate of development given the patients that recruited a higher number of oocytes per treatment were generally good responders to gonadotropin treatment (see Chapter 1) and had a larger percent of high quality blastocysts per treatment. The age of patients in group A(2˚) (<14 oocytes) was 36.4 ±0.9 years compared to 35.5 ±1.8 years in group B(2˚) (≥14 oocytes) (figure 6.2).

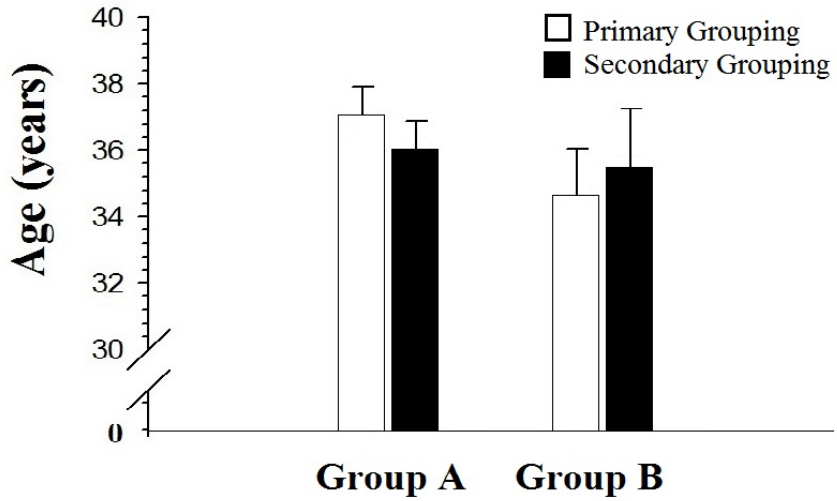



Figure 6.2. Age distribution within groups. Group A (1˚: <12 oocytes retrieved, 2˚: <14 oocytes retrieved) compared to group B (1˚: ≥12 oocytes retrieved, 2˚: ≥14oocytes retrieved. There was no significant difference of patient age in the various treatment groups.

To identify the factors influencing the variation in rate of blastocyst development per treatment, the data were analyzed with Evtree using R software, which we now discuss.

### 6.3.3 Culture Effects on Blastocyst and Cleavage Embryo Development

#### 6.3.3.1 Blastocyst Development Outcome

The success for all treatments in the study was measured with percentage of high quality blastocysts development as the final outcome measure imputed into the Evtree primary decision tree analysis. Sixty % (median) of normally fertilized oocytes had developed into high quality blastocysts. In light of this, we defined success as all treatments that had an outcome over the median (>60% high quality blastocyst development). Accordingly, the number of normally fertilized oocytes, patient age, incubator number, pH (G1 and G2 Plus), pCO2 (G1 and G2 Plus) and pO2 (G1 Plus) were all rejected as factors influencing blastocyst success (figure 6.3).

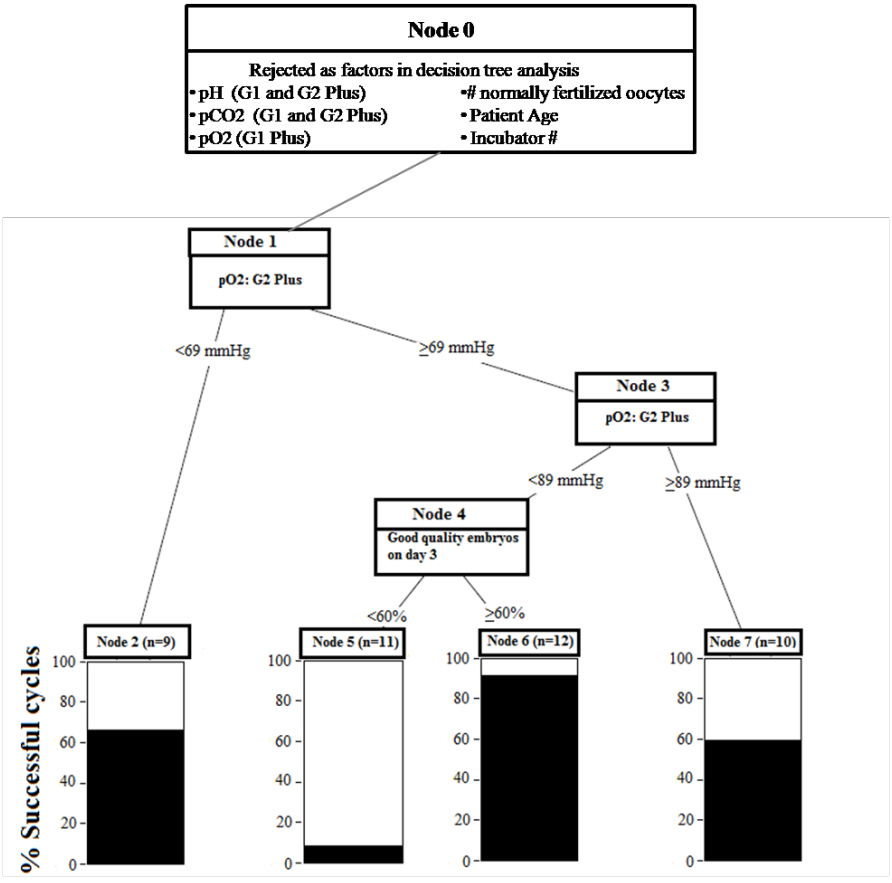



Figure 6.3. Decision tree for high quality blastocysts development. Decision tree for high quality blastocyst development, a prediction model determined by Evtree. Final graphed nodes depict successful treatments (≥60% blastocyst development, dark shading in stacked bars). Failed treatments (<60% blastocyst development) are in white shading in stacked bars. Values on the left of each bar represent the percent of successful treatments.

The $pO_2$ for the G2 Plus medium was the single contributing actionable variable that influenced blastocyst development. When $pO_2$ was <69 mmHg, there was ~70% success (Figure 6.3, node 2, n=9). When $pO_2$ was 69-89 mmHg coupled with <60% good quality embryos on day 3, treatment outcome resulted in poor blastocyst development, <10% success (node 5, n=11). Conversely, treatments that demonstrated ≥60% of good quality embryo on day 3 exhibited >90% success (node 6, n=12). This large difference in outcome was highly reliant on day 3 embryo development in this decision arm with a large variation in success (10% vs. 90%, node 11 and 12).

#### 6.3.3.2 Day 3 –Cleavage Stage- Embryo Development Outcome

A second decision tree analysis was computed to understand and identify factors that influence cleavage stage (day 3) quality. This time, the success for all treatments in the study was measured with percentage of good quality day 3 embryo development as the final outcome measure in the secondary decision tree analysis. Sixty-five % (median) of normally fertilized oocytes developed into good quality day 3 embryos. In light of this, we defined success for this analysis as all treatments, which had an outcome over the median (≥65% good quality day 3 embryo development). All variables associated with development beyond day 3 were not included in the analysis (G2 Plus medium parameters and % blastocyst development) because these parameters were a part of later embryo development and not associated with outcome of day 3 embryo quality. This model rejected pH (G1 Plus), pO2 (G1 Plus) and number of normally fertilized oocytes as factors influencing embryo success (figure 6.4). Interestingly, embryos from females <31.6 years resulted in 30% success for day 3 good quality embryo development (node 2, n=7). However, embryos from females >31.6 years and ≥14 oocytes at retrieval resulted in an 85% success (node 9, n=7).

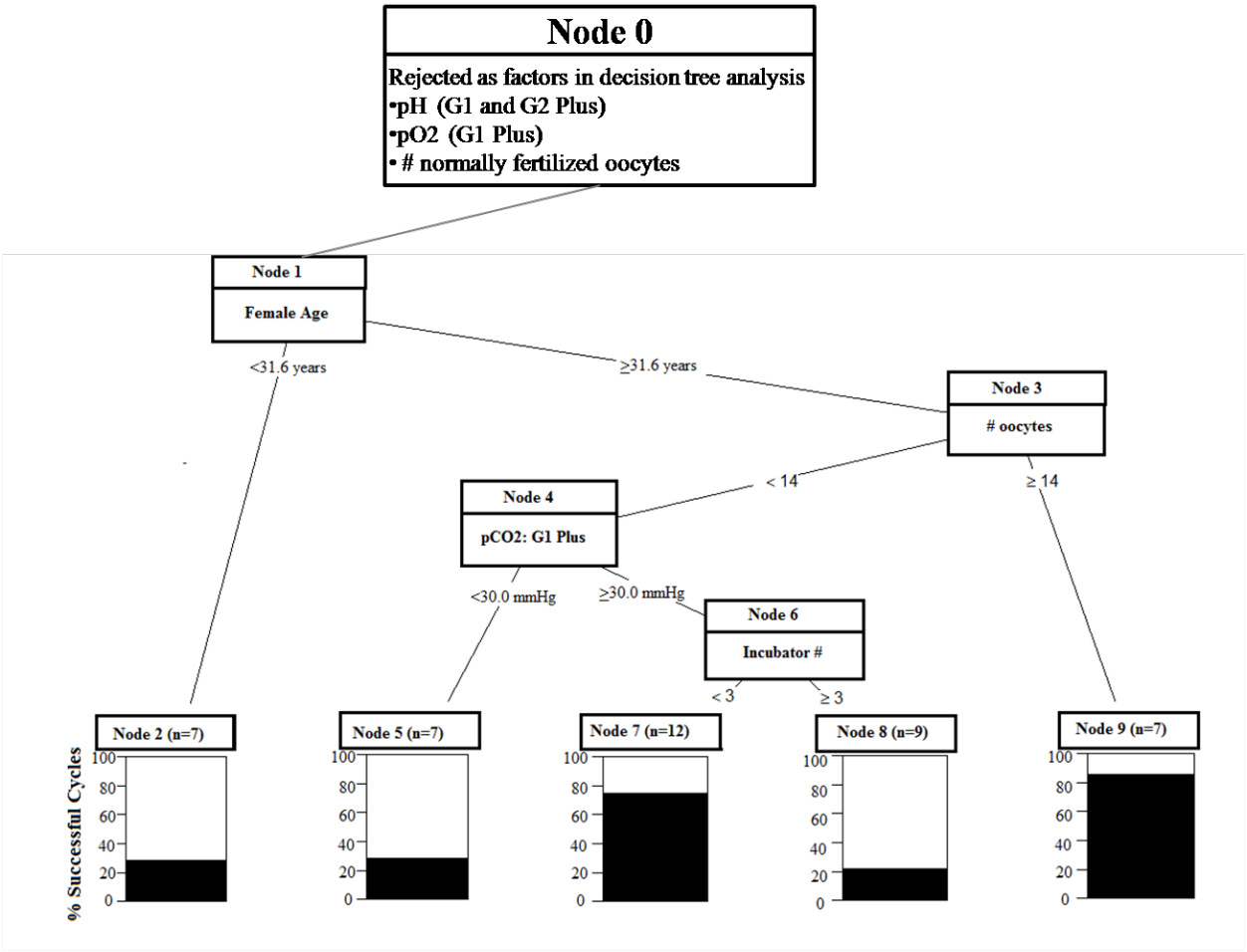



Figure 6.4. Decision tree for good quality day 3 embryo development. Determined by Evtree, final graphed nodes depict successful treatments (≥65% day 3 good quality embryo development, dark shading). Failed treatments (<65% day 3 good quality embryo development) are white. Values on the left of each bar represent the percent of successful treatments.

The medium $pCO_2$ significantly affected the outcome of day 3 embryo development from patients >31.6 years with <14 oocytes retrieved. Where $pCO_2$ was ≥30.0 mmHg, there was an 80% success for incubators #1 and #2 (node 7, n=12). However, embryos in incubator #3 had only 25% success (node 8, n=9). This identified a variation in rate of blastocysts development for embryos in incubator #3 specifically for oocytes retrieved from patients in Group A(2˚), irrespective of medium batch. Lastly, for day 3 embryos from patients >31.6 years who had ≥14 oocytes retrieved, Group B(2˚) had 80% success independent of the incubator number, pH, $pCO_2$ or $pO_2$ observed.

6.3.3.3 Accuracy Determination of Evtree Analyses

The accuracy of the primary Evtree analysis for blastocysts, determined by the receiver operating characteristic curve (ROC) (see Chapter 2), was 79% with an error rate of 21% for decision tree of blastocyst development (figure 6.5A). The ROC curve had an area under the curve (AUC) of 0.76 with 95% confidence interval (CI) of 0.63-0.88, indicating that any final node of the decision tree will result in 13% greater success than chance alone as the lowest measure and up to 38% greater success than chance alone at its highest success. The accuracy of the secondary Evtree for day 3 embryo development was 76% with an error rate of 24% (figure 6.5B). The ROC curve had an area under the curve (AUC) of 0.76 with 95% confidence interval (CI) of 0.63-0.89. This indicates that a final node on the decision tree will result in 13% greater success than chance alone as the lowest measure and up to 39% greater success than chance alone at its highest success.

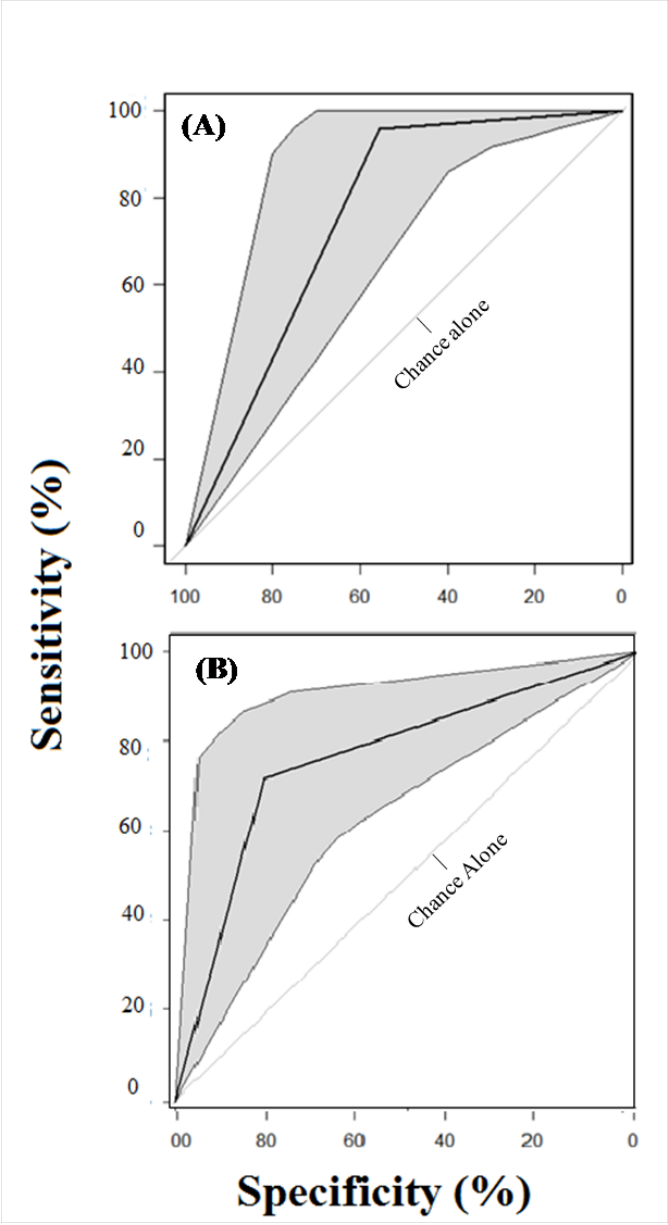



Figure 6.5. Receiver operating characteristic curve: blastocyst and embryo development. ROC Analyses of the Evtree Models for Embryo Success. True positive rate (sensitivity) is plotted against the false positive rate (1-specificity). The dark areas are the 95% confidence intervals. (A) Blastocyst development as final measure of success, (B) day 3 good quality as final measure of success.

## 6.4 Discussion

### 6.4.1 Patient Impact of Fewer Blastocysts per Treatment

Pregnancy and live birth outcomes are the standard measure of success, in which any IVF treatment resulting in a pregnancy and live birth is deemed successful (Sunderam et al. 2017; Lathi et al. 2012; Tomazevic et al. 2007). Infertility in the form of diminished ovarian reserve (DOR) or preliminary ovarian failure (POF) reduces IVF treatment success (Huang and Rosenwaks 2012; Sauerbrun-Cutler et al. 2015). Of key importance is that by this measure patients or clinicians are unable to discern whether the success was a result of a treatment, in which one pregnancy is achieved and five blastocysts may have been cryopreserved or from a treatment, in which one pregnancy is achieved but no blastocysts may have been cryopreserved for future use. In DOR and POF patients, infertility in the form of depleting ovarian reserve is advancing exponentially compared to patients with normal ovarian reserve (Levi et al. 2001). It is, therefore, crucial to ensure optimal blastocysts developmental rate for these patients maximally producing a sufficient number of blastocysts from one IVF treatment, with which the patient may complete their family building. If DOR or POF patients produce only one high quality blastocyst, used for uterine transplant, the patient may become pregnant, causing at least 9 months of delay until their next IVF treatment attempt for a subsequent pregnancy. During this lapsed time, the patient's ovarian reserve will continue depletion leading to a significant reduction in future IVF treatment success (Shahine et al. 2016; Huang and Rosenwaks 2012; Alborzi et al. 2015). Therefore, our primary concern and focus was not on pregnancy outcome per treatment, but rather on the percent of high quality blastocyst production per treatment to optimize success especially for patients with poor prognosis.

### 6.4.2 Culture Conditions, Embryos and Infertility

Excessive variability in culture conditions, however subtle, jeopardizes the rate of success of treatment measured by high quality blastocyst development. Primarily, results of the initial analysis on blastocysts development determined correlation with disparity in culture conditions observed among incubators. However, these results were dependent on cleavage stage embryo development. Interestingly, out-of-range values for $pO_2$ and $pCO_2$ of media did not negatively impact blastocysts development rate for all patients in incubators #1 and #3. The patients that were impacted were those diagnosed as affected with infertility in the form of DOR or POF as determined by the prediction model with cleavage stage embryo development as outcome.

Oxygen gradients play an important role in tissue development and proper formation of vascular structure in the developing embryo and oxygen equilibrium concentration states are inversely proportional to cell density in the embryo (Cochran et al. 2006; Hongbin Liu et al. 2009; Wale and Gardner 2016). Mitochondrial activity, as a result is hindered with variation in oxygen gradients effecting glycolysis (Saito et al. 2015; Hayashi et al. 2017). Conditions leading to lowered cellular metabolism are often induced by smoking, stress, recreational drug use, certain prescription drug use and obesity (Lindberg et al. 2016; Osadchuk et al. 2017; Denomme et al. 2017). Side-effects from drug use and obesity are linked to reproductive disorders leading to infertility, hormonal imbalance and impaired cellular function (Alvarez 2015; Beal et al. 2017; Morgante et al. 2017).

As many of the above mentioned factors influence reproductive health their conditions seem to have a commonality: roll of cellular metabolism. Cellular metabolism and respiration rely heavily on energy produced by the mitochondria used for various cellular activities including proper cell growth. Hypoxia and hypoxia-inducible factors (HIF's) can lead to unfavorable

outcomes such production of reactive oxygen species that are toxic byproducts of aerobic metabolism leading to embryonic arrest and poor embryo development in the form of apoptosis (Sagrillo-Fagundes et al. 2016; Wang et al. 2016). I speculate that in this group of patients' oxygen gradients in vivo, specifically in their reproductive organs, may be dissimilar to patients with normal ovarian reserve. Oxygen gradients in the fallopian fluid play a protective role and exert a positive effect on reactive oxygen species induced by sperm DNA damage (Belva et al. 2016; De Jonge 2017; Abdelbaki et al. 2017). However, the fallopian oxygen gradients and their direct effect on developing oocytes for patients with infertility have yet to be studied. If the conditions of DOR or POF do, in fact, result in an altered $pO_2$ gradient *in vivo*, it is not surprising that continued exposure to out-of-range *in vitro* $pO_2$ would cause harmful effects on early embryo development.

Table 6.1. Observations by medium batch across incubators

| Medium | Batch | | Incubator #1 | | | Out-of-Range by: | Incubator #2 | | | Out-of-Range by: | Incubator #3 | | | Out-of-Range by: |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G1 Plus | 5975 | pH | 7.41 | ± | 0.01* | +.007 | 7.39 | ± | 0.04 | n/a | 7.38 | ± | 0.05* | +0.04 |
| | | $pCO_2$ | 28.13 | ± | 0.52* | -3.9 | 29.97 | ± | 1.90 | n/a | 30.97 | ± | 3.36* | -1.0 |
| | | $pO_2$ | 84.67 | ± | 12.72 | n/a | 84.67 | ± | 13.25 | n/a | 78.67 | ± | 8.21* | -4.3 |
| G1 Plus | 6209 | pH | 7.35 | ± | 0.02 | n/a | NIU | | - | - | 7.33 | ± | 0.02 | n/a |
| | | $pCO_2$ | 31.68 | ± | 1.43 | n/a | NIU | | - | - | 32.60 | ± | 1.65 | n/a |
| | | $pO_2$ | 69.17 | ± | 1.25 | -14 | NIU | | - | - | 70.33 | ± | 2.75* | -12.67 |
| G2 Plus | 5979 | pH | 7.40 | ± | 0.10* | +0.06 | 7.49 | ± | 0.16 | n/a | 7.54 | ± | 0.22* | +0.14 |
| | | $pCO_2$ | 28.50 | ± | 4.95 | n/a | 25.77 | ± | 5.76 | n/a | 25.53 | ± | 6.80 | n/a |
| | | $pO_2$ | 72.00 | ± | 4.73 | n/a | 82.33 | ± | 13.84 | n/a | 89.33 | ± | 16.86 | n/a |
| G2 Plus | 6213 | pH | 7.33 | ± | 0.04 | n/a | NIU | | - | - | 7.33 | ± | 0.04 | n/a |
| | | $pCO_2$ | 30.43 | ± | 2.87 | n/a | NIU | | - | - | 31.20 | ± | 2.93 | n/a |
| | | $pO_2$ | 65.33 | ± | 1.67* | -17.7 | NIU | | - | - | 63.67 | ± | 4.49* | -19.33 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G2 Plus | 6270 | pH | 7.32 | ± | 0.00 | n/a | NIU | - | - | 7.30 | ± | 0.00 | n/a |
| | | $pCO_2$ | 33.33 | ± | 0.09 | n/a | NIU | - | - | 35.53 | ± | 0.42 | n/a |
| | | $pO_2$ | 67.00 | ± | 3.51* | -16.0 | NIU | - | - | 68.67 | ± | 1.20* | -15.33 |

NIU: Not in use.

n/a: not applicable

*Values outside of the normal acceptable ranges are bold.

Readings were performed using iStat (Abbott) analyzer on equilibrated media

### 6.4.3 Embryo Metabolism

I also evaluated culture methods to further understand this variability in $pO_2$ and $pCO_2$ readings in incubators using the same medium batch. The number of embryos per patient was divided among three culture droplets (40µl in volume each). Patients who had <14 oocytes had fewer embryos cultured per drop compared to patients with ≥14 oocytes retrieved. Therefore, culture volume was higher per embryo for group A(2˚), where more embryos, to a greater extent and more quickly influence the chemistry of the surrounding medium. This may explain why patients with ≥14 oocytes retrieved were not negatively impacted given a lower embryo to medium volume ratio. The greater collective metabolic activity of cleaving embryos, given they were from patients not highly impacted by infertility, further explains good development despite out-of-range culture conditions. More embryos per culture drop, collectively utilizing amino acids and other nutrients in the medium will lead to greater nutrient turnover (Gardner and Wale 2013; Gardner 1998; Gardner and Harvey 2015). Embryos created from oocytes or sperm that are exposed to primary stressors, such as patient age, genetics, etiology, etc., are at high risk of lowered treatment success. Subjecting these embryos to secondary stressors, such as out-of-range culture conditions causes vulnerability for embryonic development in the form of morphology, phenotype and genotype (Gardner and Kelley 2017).

Increased respiration rate is associated with viability in cleavage stage embryos (Tejera et al. 2012; Wakeland et al. 2017; Yang et al. 2016). Incubators are set to low oxygen (5% $O_2$) to mimic presumed physiological conditions in part because atmospheric oxygen levels will result in oxidative stress in the form of free radicals causing lower numbers of high quality blastocysts (Bedaiwy et al. 2004; Kelley and Gardner 2016; Y. Yang, Xu, et al. 2016). Glucose, the primary nutrient of the blastocyst, is metabolized through aerobic glycolysis and oxidation (Gardner and Harvey 2015; Perkel and Madan 2017). Oxygen partial pressure surrounding the blastocysts ensures proper regulation of metabolic pathways during development, which ultimately leads to successful implantation and live birth. Severe hypoxia ($\leq$3.5%) will reduce blastocyst development (Fawzy et al. 2017; Guo et al. 2014). Therefore, it is critical that $pO_2$ is within normal range for culture of embryos *in vitro*. Additionally, it is critical that $pCO_2$ is within normal range for G1 Plus (bicarbonate buffered) primary culture medium, to ensure optimal pH, in which essential amino acids, salts and nutrients are biochemically in an environment where they can be appropriately be utilized by the developing embryo. Without this balance, embryo development will be hindered resulting in lower quality blastocysts.

### 6.4.4 Implications to Patients of Present Study

Lowered outcome success leads to emotional and financial implications on patients since each round of IVF treatment can cost ~$12,000. While some patients are financially secure, others may elect to barrow funds either from financial institutions or personal loans from family and friends. Lack of success or low number of high quality blastocysts per treatment, especially for the patients that have borrowed funds, can lead to emotional trauma with heavy implications on patient/partner relationship. Although patients are informed that failure is a possibility prior to

treatment, coping with this negative outcome can be a grief-driven and burdensome process. The clinical staff and physician directly interact with these patients to assist medically with this hardship in the form of scientific explanations for individualized treatment failure and success odds of future attempts.

I accepted my hypothesis that subtle variation in incubator culture conditions can lead to poor blastocyst development rate for certain infertility patients who we identified to have low ovarian reserve. Additionally, I did not find that the acceptable rages of pH, $pCO_2$ and $PO_2$ are too broad. I have instead identified greater influence on blastocyst developmental potential due to environmental influence such as subtle variations in incubator performance (producing out-of-range culture values for $pCO_2$ and $pO_2$) and the interaction with oocytes predisposed to primary *in vivo* stressors, previously described. The treatments impacted by low $pCO_2$ on days 1-3, mainly resulted in no success, demonstrating irreparable effects of early phase culture in the subset of patients with DOR or POF. Expansion of this study using randomization and blinding to embryologists in a multi-center setting would be well worth investigating. A multi-center study will be significant to broaden industry understanding on IVF culture systems and perhaps allow for individualized culture conditions based on patient diagnosis.

## 6.5 Conclusion

It is the ultimate responsibility of embryologists performing human *in vitro* fertilization to provide optimal culture conditions for all gametes and embryos. Subtle variability in culture conditions may occur across media batches, even when levels of $O_2$ and $CO_2$ concentrations within incubators are held within acceptable ranges. Maintenance of chemical composition in the medium is critical in optimization of embryo development. I recommend more frequent quality assurance

studies within laboratories to identify intra-laboratory occurrences, which may impact treatment outcome and ensure that embryos and oocytes from patients with DOR and POF are cultured in incubators with minimal variability especially in since they present as high risk of being negatively impacted in the form of decreased rate of high quality blastocysts development by high $pO_2$ and low $pCO_2$. Incubators with minimal variability should be reserved for patient presenting with diminished ovarian reserve or preliminary implantation failure for optimal outcome since all other patient treatments were not impacted. The current study reflects the influence of environmental factors on the overall rate of high quality blastocysts development per patient treatment. Frequent assessment and analysis of the culture system and laboratory equipment performance are critical to the overall patient treatment optimization and success.

# CHAPTER 7

# FINAL CONCLUSIONS

## 7.1 Mammalian Embryos and Blastocysts

From the moment of fertilization, mammalian embryos initiate a cascade of molecular pathways, each as a response to its development and environment. An embryo develops from a single totipotent cell into its extraordinarily complex system of modules that allows anatomical changes in the organism without one module necessarily interfering with other modules. The embryo is complex in that the process of embryogenesis and builds diverse layers of complexity including transcription programs, which propagate information from the molecular level to the tissues and organs later in development (Edelman et al. 2010; Chitwood et al. 2017; Li et al. 2017). Additionally, the embryo builds complexity to its individual systems by linked components allowing it to function as a whole system comprising of smaller systems. As the embryo grows, cells differentiate (e.g., inner cell mass, trophectoderm) according to chemical gradients and transcriptional factors. Post-cavitation, the mammalian embryo (now referred to as blastocyst) is adaptive with the capacity to change its behavioral responses to the environment for successful growth. The adaptability of blastocysts allows for expression of various phenotypes for one genotype relative to its environment. Due to the emergent behaviors and responses dependent upon the interactions of various factors in the blastocysts' environment the study of embryo adaptability remains complex, yet intriguing.

### 7.1.1 Adaptability with Limitations

The natural environment for development of the embryo and blastocyst are the fallopian tubes and the uterus. The practice of *in vitro* fertilization (IVF) takes advantage of one of the

adaptive attributes of the blastocyst capability to develop outside the body, in culture. The blastocyst responds to environmental cues by demonstrating a high degree of developmental plasticity in the form of metabolic modulation, gene expression and cell division rate (Soundararajan et al. 2015; Price et al. 2003; Denver and Crespi 2006; Agrawal 2001). The success of blastocysts grown in culture is ultimately measured by rate of live births per uterine blastocyst transfer (Sunderam et al. 2017; Farquhar et al. 2013; Tomazevic et al. 2007; Staessen et al. 2008; Heitmann et al. 2013). The growing cleavage stage embryos and eventual blastocysts demonstrate plasticity and are able to react to an external input or injury by altering its state or fate (Pollard et al. 2017; Bedzhov et al. 2014).

I challenged blastocyst plasticity in the graft transplant study (Chapter 3) by temporarily removing calcium, necessary for cell-to-cell adhesion, from blastocyst culture. I identified another form of adaptive limitations in embryogenesis. In the mouse, induced and stage dependent loss of functionality in cell adhesion occurs post-calcium removal from blastocyst culture (Gardner and Simón 2017; Bronner 2001; Nagy et al. 2012). The resulting decline in growth was indicative of the impact of environmental factors, even in the form of minute exposures, which lead to a cascade of chemical events altering the fate of the blastocyst (Yang et al. 2017; Lim et al. 2016). In this study, blastocysts were unable to fully recover from the induced stress caused by temporary disruption of bonds associated with gap junctions in the trophectoderm. The blastocysts that were exposed to calcium-free solution on day-5 compared to day-4 demonstrated adaptability in the form of partial re-expansion of the blastocoel.

Limitations of the blastocysts relative to their environment were also identified in Chapter 4. In this study, pre-implantation genetic screened euploid blastocysts that exhibited organized development were high quality blastocysts. These were selected for uterine transfer. Surprisingly,

some “euploid” blastocysts resulted in spontaneous abortion prior to 20 weeks of gestation, specifically when array comparative genomic hybridization and small nucleotide polymorphism techniques were used. It is likely that a contributing factor in the early miscarriage was due to undetected mosaicism or segmental aneuploidy. Previously discussed, array comparative genomic hybridization and small nucleotide polymorphism array techniques did not recognize or report mosaicism in biopsies analyzed. This indicates the embryonic adaptability limitations that are presented with challenges of mosaic cell lines early in development. A pregnancy, confirmed with elevated beta-human chorionic gonadotropin levels in blood serum 10 days post euploid embryo transfer, followed by spontaneous abortion is characteristic of obstacles associated at the fetal genome level. Despite consisting of more than one cell lineage, the mosaic embryo, in theory, can overcome conditions that prevent implantation, however, it’s limitation is reached when it could no longer grow into a viable fetus and the result is often a spontaneous abortion or a miscarriage. This limitation results in early pregnancy loss when the mosaic embryonic genome fails to grow into a chimeric organism.

Embryonic adaptability was identified in Chapter 6. My findings indicated that cleavage embryos can adapt to subtle differences in culture pH and partial pressures of $CO_2$ and $O_2$. Day 3 embryos cultured in incubator 3, which resulted in slight out-of-range values for culture conditions, were able to adapt to the change in their environment when not predisposed to primary stressors leading to infertility (90% success), like preliminary ovarian failure or diminished ovarian reserve. Under the same culture conditions, day 3 embryos of individuals with lower numbers of oocytes retrieved (<14) were not able to adapt as well (~20% success) (Chapter 6). Despite plasticity; the embryos and blastocysts have their limitations in the form of conditional adaptation.

The blastocyst can also demonstrate adaptability in the form of chimeric growth. Cheatham

et al. (2009) produced chimeras by fusing the embryonic cells of the two mice strains to construct a mouse with the desired knock out gene. The individual cells, obtained from two different lineages, when placed within proximity in the same blastocyst demonstrated adaptability and continued development relative to their neighboring cells into a complete chimeric organism. Cell specification, developmental signaling and cell migration are influenced, in part, by chance fluctuations by transcriptional factors, paracrine factors and receptors produced at various times throughout development of the blastocyst (Breau and Schneider-Maunoury 2015; Ruvinsky et al. 2009; Basson 2012). Together with its individual components the developing blastocyst has the ability to act independently, creating sub-specialized cell types and can act as a holistic organism related to its neighboring modules. In this way, the blastocyst is both complex and adaptive relative to the theory of systems biology (Brodland 2015; Plouhinec and De Robertis 2007; Edelman et al. 2010). Albeit, the blastocyst is endowed with adaptive functionality to environmental challenges, the burden to simultaneously maintain homeostasis is considerable.

### 7.1.2 Homeostasis – *in Vivo* and *in Vitro*

Homeostasis, whereby the embryo or blastocyst maintains a stable equilibrium with its internal components regardless of the conditions found in its environment, is maintained in various ways (Swain 2010; Lane and Gardner 2000; Swain et al. 2012). Critical to development is the maintenance of ionic homeostasis carried out by regulation of intercellular pH, calcium ions, magnesium ions and phosphate ions (Swain 2012; Lane and Gardner 2000; Dubyak 2004). The maintenance of a balance of ions results in proper cell development and function. Embryonic cells utilize calcium/potassium pumps embedded in the lipid bi-layer to control the amount of calcium and potassium concentrations within the cell cytoplasm and maintain homeostasis (Bavister 2012;

Watson and Barcroft 2001).

Homeostasis for embryos and blastocysts was observed in my studies. As an example, in the cell graft study (Chapter 3), cells were removed from one blastocyst and transferred to a sibling blastocyst. This study takes into account the ability of the blastocyst to maintain homeostasis by apoptotically removing damaged cells ablated by the Saturn laser during the graft transplant procedure. It also shows the blastocysts capacity for homeorhesis in the form of cell graft annealing to sibling trophectoderm cells for continued development. As the blastocyst recovered from the procedure, the cells functioned independently to maintain cell-to-cell interaction of gap junctions by the removal of damaged cells (apoptosis) and attachment of neighboring cells (sibling grafts) for survivability and continued viability. Blastocysts were able to successfully maintain homeostasis post-treatment under day 4 and day 5, stage-dependent and modified Human Tubal Fluid conditions in this study. Blastocysts completely re-expanded their blastocoelic cavity, demonstrated symmetry in the trophectoderm and incorporated and integrated sibling graft cells (homeorhesis) when treatment was with modified human tubal fluid (mHTF) (E-cadherin-catenin bonds not disrupted). The cell ablation and graft transplant procedure did not result in decline in blastocyst morphology or reduce viability. However, the disruption of cell adhesion (calcium removal) disrupted homeostasis, from which the blastocyst could not recover, exhibiting their limitations.

Homeorhesis takes the change occurring within an organism and within the space-time continuum to describe the constant interactions of the organism with its changing environment as necessary for the organism's well being and adaption (Winslow 2017; Gilbert 2000). My studies combined both the theory of homeostasis and homeorhesis by evaluating how the embryo stabilizes its different components while maintaining development. In Chapter 6, I studied the

effects of culture conditions on embryo development and the rate of blastocyst formation with respect to the small shifts in pH, partial pressures of carbon dioxide and oxygen throughout culture. As an example, in this study, incubator #1 had out-of-range pH by +0.07 in the primary culture media (G1 Plus). Although the extracellular pH was higher than expected, embryos were able to maintain homeostasis and homeorhesis by demonstrating blastocyst development. The predictive modeling analysis determined incubator #1 was insignificant as a factor that influenced a decline in blastocysts formation, based on pH differences in culture.

## 7.2 Limitations of *In Vitro* Fertilization

### 7.2.1 Rate of Blastocyst Development

*In vitro* fertilization treatment outcomes, viewed as rate of blastocysts production per treatment, vary among patients. Female patients <35 years old are expected to have a higher rate of blastocyst development compared to the patients >40 years (Cohen et al. 2015; Meldrum et al. 2016; Tomazevic et al. 2007). In Chapter 5, I identified a strong correlation of negative outcome - rate of blastocysts development- in individuals characterized as “good responders” presenting with high AMH values ( ≥3.1 ng/ml) specifically when exposed to high gonadotropin doses (>3325IU) during stimulation. According this criteria, the rate of success was 0% (n=11) as determined by the decision tree (Chapter 5). Although unexpected, this outcome can be improved by controlling secondary stressors to influence outcome as described in the prediction model.

Age, undoubtedly, is an important factor and a primary-stressor for infertile patients. However, in Chapter 6, a greater impact on blastocyst developmental potential was identified when age was coupled with low ovarian reserve. Ovarian reserve depletes for women at variable rates. Therefore, all 40 year old females will not present with diminished reserve. For example, a female

who is 40 years and diagnosed with polycystic ovaries is anovulatory and presents with a normal ovarian reserve despite age. This patient is expected to have better prognosis than a patient of the same age with elevated basal FSH, ovulatory and low ovarian reserve (Minooee et al. 2017; Morgante et al. 2017; van der Meer et al. 1994). Cohen *et al.* (2015), did not consider the magnitude of impact resulting from coupling gametes predisposed to primary stressors (i.e. DOR) with multiple secondary stressors (i.e. high gonadotropin doses during ovarian simulation and slight out-of-range culture $pCO_2$ and $pO_2$ for embryology). In my studies, coupling of prolonged exposure to multiple conditions collectively result in lowered blastocyst developmental potential (see Chapters 5 and 6) by depreciating the ability of the blastocyst to adapt to its environment. Namely, Chapter 6, blastocysts impacted by low $pCO_2$ on days 1-3, mainly resulted in no success for oocytes retrieved from diminished ovarian reserve patients, as mentioned above.

On average, 40% of fertilized oocytes develop into blastocysts (Zhao et al. 2012; Tomazevic et al. 2007; Heitmann et al. 2013), which is a demonstration of their adaptability and an indication that blastocysts can thrive in culture with greater success outcome if culture is individualized per patient. Individualized culture requires evaluation of primary stressors such as patient age, genetics, etiology, etc. In all four of my studies, I identified factors that influence the low rate of blastocyst formation per IVF treatment. Extended research analyzing specific metabolic events, which lead to attretic, arrested and poorly developed blastocysts are well worth conducting. Furthermore, parallel study of variables resulting in high quality blastocysts will allow insight and aid in identification of key factors in the molecular processes, which result in favorable development. Clinicians routinely individualize gonadotropin doses by patient diagnostics (van Tilborg et al. 2016; Andersen et al. 2017; Oudshoorn et al. 2017). The future of IVF will very likely involve individualized culture conditions for complete optimization of IVF.

### 7.2.2 Abandoned Blastocysts

Controlled ovarian hyperstimulation results in the formation of multiple embryos and blastocysts per cycle. Patients struggle with the disposition options available for excess unused blastocysts. Once family planning is completed and patient and partner have reached an agreement regarding final disposition of excess cryopreserved embryos, the laboratory is notified. One of the following options is usually selected by the genetic parents: any blastocysts remaining in cryopreservation storage are discarded (warmed to room temperature and disposed of as medical waste), donated to research or anonymously donated to an infertile couple. If the patient and partner have not agreed on final disposition of embryos and refuse to communicate with the clinic or laboratory regarding a decision, the embryos are considered abandoned. IVF laboratories are still facing challenges on how to deal with this dilemma. I strongly encourage the use of the decision tree in Chapter 5, which results in minimizing creation of excess blastocysts for good prognosis patients presenting with normal ovarian reserve and maximizing this outcome for patients presenting with low ovarian reserve. This tailored approach will collectively lower the creation of surplus blastocysts. Ultimately, this approach will lessen the patient emotional burden associated with the final disposition of cryopreserved embryos. Additionally, laboratories should restructure IVF protocols by the use of predictive modeling analysis using their own treatment outcomes. I recommend analysis conducted every 2 years. Findings of the prediction model specific to site can be compared against Figure 5.1 to ensure reproducibility of the predictive model. Standardized ovarian stimulation protocols based on the decision tree analysis can be created, which explicitly state medication doses, days of stimulation, etc. to offer the highest standards of IVF treatment to infertility patients.

### 7.2.3 Pre-implantation Genetic Screening

Preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS) have been used in IVF to detect unbalanced chromosome copy numbers or single gene disorders in blastocysts (Zhu et al. 2014; Aleksandrova et al. 2016; Dahdouh et al. 2015; Fiorentino et al. 2014; Harper and Harton 2010; Li et al. 2014). I incorporated three of the widely used methods of blastocyst screening offered to patients undergoing IVF in Chapter 4: array comparative genomic hybridization (aCGH), small nucleotide polymorphism (SNP) array, and next generation sequencing (NGS). No significant difference in pregnancy outcome resulted from euploid-screened blastocysts by any of the three screening methods in patients 35-37 years of age. Importantly, patients < 34 years did not benefit from screening blastocysts using any of the three methods identifying limitations to PGS (Chapter 4). It would be well worth investigating alternative forms of screening such as methylation patterns in the embryonic genome and assessment of mitochondrial DNA in addition to PGS for greater insight on blastocyst genomics. Identification of anomalies other than chromosome aberrations in the genome can allow optimization for blastocysts selection for uterine transfer. Ultimately, this information will aid in the selection of blastocysts with the highest implantation success leading to improved live birth rates.

### 7.2.4 Evolution of IVF: Inclusive Embryology

Ovarian stimulation during IVF treatment often recruits a large number of oocytes. The general practice is to identify and utilize the blastocysts that are well developed at the end of the treatment by excluding others that demonstrate arrested or attretic morphology. The technique of preimplantation genetic screening presents further exclusion of blastocysts that exhibit the aforementioned high grade morphology. This exclusion is applied by eliminating aneuploid

blastocysts for uterine transfer. Few to no solutions offer potential methods to include poorly developed blastocysts. Consequently, they are excluded from selection for uterine transfer or cryopreservation procedures. Approaching infertility treatment with an inclusion method will assist in achieving higher success for some patients. In Chapter 3, I presented findings, in which I applied a novel technique focused on rectification of this issue in a mouse model: graft transfer embryonic stem cells (GT-ESC). Specifically, blastocysts that develop a well formed inner cell mass but a poorly formed trophectoderm, or vice versa, are blastocysts that would benefit most from this technique. Under the right culture conditions, blastocysts are able to integrate donated cells from sibling blastocysts into their own TE and exhibit continued development. Given this initial success, future studies conducted to examine live birth outcome resulting from sibling graft recipient blastocyst in animal models are well worth investigating. In addition to live birth rates, studies investigating expression rate of E-Cadherin during various stages of blastocyst development during and post graft transplant and offspring health studies from use of such blastocysts will pave the way for eventual use of this technique in human IVF.

## 7.3 Cost Factors

### 7.3.1 Financial Burden

Monetary costs (to patients or insurance companies) are inevitably associated with IVF treatment. However, my findings indicate methods that can lower this burden for patients. Importantly, analysis of PGS and pregnancy outcome determined blastocysts from individuals of <34 years of age do not benefit from screening. Patients should be counseled appropriately to minimize unnecessary biopsy procedures on blastocysts and to minimize costs associated with such procedures. Additionally, the predictive model analysis in Chapter 5 provides maximal

discrimination between subgroups to determine the values associated with each node on the decision tree, referred to as a 'split rule', while maintaining high homogeneity within each subgroup. The resulting split rules provide a robust decision tree, which should be used as a resource to minimize medication dose (total IU), limiting financial burden while maximizing outcome of high quality blastocyst development per treatment. Finally, limiting the number of surplus blastocysts created per treatment can lower the patient's financial burden with respect to ongoing laboratory storage fees associated with maintaining and monitoring blastocysts in cryopreserved state.

### 7.3.2 Emotional Burden

Diminished ovarian reserve (DOR) and preliminary ovarian failure (POF) patients represent approximately 30% of the IVF treatments in the United States (Cohen et al. 2015; "ART Report 2013-CDC" 2015). These patients are at high risk of developing mosaic blastocysts (Capalbo et al. 2017; Munné et al. 1993; Sermon et al. 2016). Proper counsel and screening of blastocysts by NGS will identify errors beyond whole chromosome or large segmental deletions or duplications. NGS, identifies mosaicism, thereby reducing the rate of spontaneous abortions and ultimately increasing the rate of sustainable pregnancy in individuals with advanced maternal age. NGS use for patients >40 years will lessen the emotional burdens that present with miscarriages (Chapter 4).

I also identified that AMH values are reliable indicators of blastocyst developmental potential compared to basal FSH values (Chapter 5) that can be used as a tool to properly counsel patients seeking IVF treatment. I confirmed that patients with DOR ($\leq$12 mature follicles at trigger) , POF or poor prognosis exhibit a high rate of good quality blastocyst development per treatment,

which positively correlates with increasing levels of gonadotropins inversely related to individuals with a higher number of mature follicles at trigger (>12). Utilization of the decision tree for blastocysts optimization will further lessen the emotional burden associated with negative outcomes for these poor prognosis patients.

## 7.4 Final Thoughts

The evidence and understanding of embryology and mechanisms of self-correction of DNA errors in early blastocysts are still not well understood. It is important to distinguish between organismal survival versus thriving growth. Considering the interactions between many of the variables and factors associated with an IVF treatment, blastocysts development outcome can be improved with the use of decision trees presented in my studies from stimulation to culture and ultimately, blastocysts' rescue. As the era of IVF advances to higher technologies (i.e. NGS and graft transplants) the burden is on embryologists and clinical researchers to accurately assess their findings in a robust inclusive manner accounting for interactions of all variables simultaneously. With the advancements in the field statistics using artificial intelligence, research findings are even stronger and can produce greater success in the IVF field with accurate predictability. In the end, the goal is to optimize care and treatment and provide elite conditions for the success of the patient.

# APPENDIX A

## EMBRYOTECH RECOMMENDED THAWING INSTRUCTIONS

EMBRYOTECH™ laboratories

# Product Thawing Instructions

**Because of the delicate nature of the ova and/or embryos, it is important that all instructions be followed.**

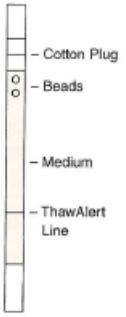
## ThawAlert™

- To ensure that each shipment of ova and/or embryos arrives at the proper temperature, Embryotech™ has devised a unique and simple method - the ThawAlert™ straw.
- With every shipment of ova and/or embryos, a ThawAlert™ straw will be placed in a specially designated cane.
- Each ThawAlert™ straw has been prepared in such a way that two metal beads are frozen in place just below the cotton plug and above a line marked on the straw.
- Fill the shipper with liquid nitrogen.
- Locate and remove the ThawAlert™ straw and check the position of the two metal beads (see Figure 1).
- If the appropriate temperature was maintained inside the shipper during transit, the beads will be located above the ThawAlert™ line marked on the straw.
- If the internal temperature of the shipper was compromised during transit, the beads will be located below the ThawAlert™ line marked on the straw, indicating premature media thaw.

**IMPORTANT: If you find a ThawAlert™ straw with a bead position below the ThawAlert™ line, please call us at (978) 373-7300 or (800) 673-7500.**

Figure 1

**ThawAlert™ Straw**




## Thawing Instructions

1. Remove straw from storage tank and expose to room temperature air for 2-3 minutes.
2. Next place straw in a 37°C water bath for 1 minute.
3. Remove straw from water bath and wipe it dry.
4. Expel the contents of the straw using the following procedure:

a) Remove handle

b) Using a pair of scissors (see figure 2), cut the straw between the lower heat seal and the column of media.

c) Make a second cut to bisect the cotton plug at the PVA section.

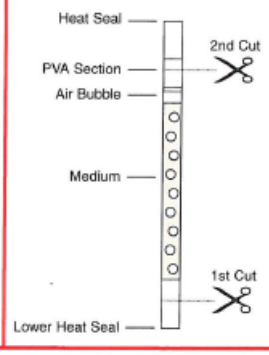
d) In a petri-dish using the stylet, push down on the remaining cotton plug, expelling the contents of the straw into a drop of *M2, mHTF or similar Hepes buffered media.

5. Immediately rinse the cells through a minimum of 2 additional drops of a Hepes buffered media pH 7.0 - 7.4 at room temperature.
6. Allow cells to rehydrate for 10 minutes in the final rinse droplet.
7. The ova or embryos are now ready for use.

*M2: provided at no extra charge on request.

Figure 2

**Frozen Ova or Embryo Straw**




140 Hale Street Haverhill MA 01830 USA tel 978.373.7300 fax 978.373.7399 info@embryotech.com www.embryotech.com

# APPENDIX B

# VITROLIFE G1-PLUS CERTIFICATE OF ANALYSIS

Vitrolife

# CERTIFICATE OF ANALYSIS

## G-1™ PLUS

| REF | CONTENT | LOT | EXPIRY DATE |
|---|---|---|---|
| 10128 | 1x30 mL | 506716 | 2017-11-21 |

RCVD 8/31/17

**INDICATION FOR USE**
Medium for culture of embryos from the pronucleate stage to day 2 or day 3.

**DESCRIPTION**
Bicarbonate buffered medium containing hyaluronan and human serum albumin.

**APPLICATION**
For use after equilibration at +37°C and 6 % $CO_2$.

Oscar Perez. Ph.D., (HCLD/ELD)
Laboratory Director
Reviewed date: 10-26-17

**RAW MATERIALS**
All raw materials are tested and evaluated by stringent quality control procedures.

**COMPOSITION**

| | | | |
|---|---|---|---|
| Alanine | Alanyl-glutamine | Asparagine | Aspartate |
| Calcium chloride | EDTA | Gentamicin | Glucose |
| Glutamate | Glycine | Human serum albumin* | Hyaluronan |
| Lipoic acid | Magnesium sulphate | Methionine | Potassium chloride |
| Proline | Serine | Sodium bicarbonate | Sodium chloride |
| Sodium citrate | Sodium dihydrogen phosphate | Sodium lactate | Sodium pyruvate |
| Taurine | Water for injection (WFI) | | |

*Pharmaceutical infusion-grade for medical use, free from HBV, HCV and HIV.

| PRODUCT PROPERTIES | SPECIFICATION | RESULT |
|---|---|---|
| pH (at +37°C and 6 % $CO_2$ atmosphere) | 7.27±0.07 | 7.27 |
| Osmolality [mOsm/kg] | 280±5 | 282 |
| Sterility Assurance Level (sterile filtration) | 1E-3 | 1E-3 |
| Bacterial endotoxins (LAL assay) [IU or EU/mL] | <0.25 | <0.25 |
| Mouse embryo assay (1-cell)[% expanded blastocyst within 96h] | >=80 | >=80 |
| Mouse embryo assay (1-cell)[blastocyst cell number within 96h] | No statistical difference [1] | PASS |

[1] Mean cell number of the test group is statistically compared to the control group. The mean of the test group must not be statistically different (P>0.05) to the control to constitute a Pass.

2017-07-26
Camilla Johansson
Quality Control Manager

This document has been produced electronically and bears no signature. The data can be verified by our QC-Department. For more information please see our internet homepage: www.vitrolife.com

# APPENDIX C

# VITROLIFE G2-PLUS CERTIFICATE OF ANALYSIS

Vitrolife

# CERTIFICATE OF ANALYSIS

## G-2™ PLUS

| REF | CONTENT | LOT | EXPIRY DATE |
|---|---|---|---|
| 10132 | 1x30 mL | 506720 | 2017-11-22 |

RCVD 8/31/17 BB

**INDICATION FOR USE**
Medium for culture of embryos from day 3 to the blastocyst stage.

Oscar Perez. Ph.D., (HCLD/ELD)
Laboratory Director
Reviewed date: 12-Sep-17

**DESCRIPTION**
Bicarbonate buffered medium containing hyaluronan and human serum albumin.

**APPLICATION**
For use after equilibration at +37°C and 6 % CO2.

**RAW MATERIALS**
All raw materials are tested and evaluated by stringent quality control procedures.

**COMPOSITION**

| | | | |
|---|---|---|---|
| Alanine | Alanyl-glutamine | Arginine | Asparagine |
| Aspartate | Calcium chloride | Calcium pantothenate | Cystine |
| Gentamicin | Glucose | Glutamate | Glycine |
| Histidine | Human serum albumin* | Hyaluronan | Isoleucine |
| Leucine | Lysine | Magnesium sulphate | Methionine |
| Phenylalanine | Potassium chloride | Proline | Pyridoxine |
| Riboflavin | Serine | Sodium bicarbonate | Sodium chloride |
| Sodium citrate | Sodium dihydrogen phosphate | Sodium lactate | Sodium pyruvate |
| Thiamine | Threonine | Tryptophan | Tyrosine |
| Valine | Water for injection (WFI) | | |

*Pharmaceutical infusion-grade for medical use, free from HBV, HCV and HIV.

| PRODUCT PROPERTIES | SPECIFICATION | RESULT |
|---|---|---|
| pH (at +37°C and 6 % CO2 atmosphere) | 7.27±0.07 | 7.22 |
| Osmolality [mOsm/kg] | 280±5 | 281 |
| Sterility Assurance Level (sterile filtration) | 1E-3 | 1E-3 |
| Bacterial endotoxins (LAL assay) [IU or EU/mL] | <0.25 | <0.25 |
| Mouse embryo assay (1-cell)[% expanded blastocyst within 96h] | >=80 | >=80 |
| Mouse embryo assay (1-cell)[blastocyst cell number within 96h] | No statistical difference [1] | PASS |

[1] Mean cell number of the test group is statistically compared to the control group. The mean of the test group must not be statistically different (P>0.05) to the control to constitute a Pass.

2017-07-26
Camilla Johansson
Quality Control Manager

This document has been produced electronically and bears no signature. The data can be verified by our QC-Department. For more information please see our internet homepage: www.vitrolife.com

# APPENDIX D

# ANIMAL TISSUE USE APPLICATION

RECEIVED
SEP - 2 2015
RESEARCH COMPLIANCE

# Animal Tissue Use Application

University of North Texas
Institutional Animal Care and Use Committee

## TISSUE RECIPIENT

| | |
|---|---|
| **Title of Project:** "Developmental Implications of Vertebrate Blastocyst Disassembly and Reassembly" | **Filled out by IACUC office only** 15019 |
| **Principle Investigator:** Warren Burggren | **Protocol #:** |
| **Department:** Biological Sciences | **Approval Date:** |
| **Campus Mail Address:** 1155 Union Circle #305220 | **Expiration Date:** |
| **Telephone:** 940-367-2882 **Date:** 8/14/51 | |
| **Email:** burggren@unt.edu | |

## TISSUE SOURCE

1. If tissue is supplied from a UNT investigator, provide protocol number:

| | |
|---|---|
| **Vendor/PI:** Jackson Laboratory | **Telephone:** 207-288-6000 |
| **Mailing Address:** The Jackson Laboratory 600 Main Street Bar Harbor, Maine 04609 USA | **Email:** No email available. |

2. Species: Mouse
3. Tissue type (brain, skin, eyes, blood, etc): 2 cell stage fertilized embryos
4. Quantity: 400
5. Mode of transport: Air/Ground Carrier

## TISSUE USE

1. PI/Collaborators/ Laboratory Personnel

Indicate by completing the following table the qualification of investigators, professional technical, or student personnel who will be overseeing or actually performing experimental procedure(s) with animal tissue.

| Last Name, First Name | Position | EMPL ID | Emergency Contact Phone Number |
|---|---|---|---|
| Burggren, Warren | Professor | 10000258 | 940-367-2882 |
| Sadruddin, Sheela | PhD Candidate | Student # 10241517 | 871-891-8540 |
| | | | |
| | | | |
| | | | |
| | | | |

2. Will these tissues be used in live animals? ☐ Yes ■ No
If YES, protocol number: _____

3. Will these tissues be exposed to any hazardous materials (radioisotopes, biohazards, or chemical hazards)? ☐ Yes ■ No
If YES, list the name and properties (half-life, zoonotic potential) of hazards: ____

4. Where will experiments be conducted? Bldg & Rm #: LSCB 247
Are these laboratories used for live animal experiments? ■ Yes ☐ No
If YES, provide protocol number(s): Only Four PIs in this lab

5. Briefly describe the use of the tissues:

We will receive two-cell stage frozen mouse embryos from multiple vendors, and will leave them frozen in liquid nitrogen until their removal. Upon removal, they will be placed in a CO2 incubator and their development followed after minor experimental manipulation - typicaly removal of some cells. The fertilized eggs will then be allowed to grow up to about 80 cell blastocyst stages, at which point they will be discarded according to standard safety protocols for disposal of tissue (although they will still be microscopic at this stage).

**PI ASSURANCE**

I assure that all slaughterhouse materials used are obtained from USDA inspected facilities.

I assure that no additional animals are euthanized under this protocol nor are any additional procedures carried out on these animals prior to their death, for the purpose of the applicant's research.

I assure that all personnel have been trained on the safe handling of animal tissues.

I assure that tissues are not from an endangered species and do not violate the Migratory Bird Act.

As Principle Investigator, I am aware that I have ultimate responsibility for the proper use of animal tissues and associated chemical, radioactive, and biosaftey hazards. I agree to adhere to all federal, state, and local laws and regulations governing the use of fresh animal tissues in research. I further assure the University of North Texas Animal Care and Use Committee that the minimal number of animal tissue will be used for the project. I have carefully considered and concluded that no reasonable alternative to the use of animal tissue could be applied to this project, and that this project is not an unnecessary duplication of any previously published work.

Warren W. Burggren — 8/14/15
Principle Investigator/Course Director — Date

# REFERENCES


"2015 Assisted Reproductive Technology National Summary Report." Accessed October 28, 2017. https://www.cdc.gov/art/pdf/2015-report/ART-2015-National-Summary-Report.pdf.

Abdalla, H. 2004. "An Elevated Basal FSH Reflects a Quantitative Rather than Qualitative Decline of the Ovarian Reserve." *Human Reproduction* 19 (4):893–98. https://doi.org/10.1093/humrep/deh141.

Abdalla, H., and M. Y. Thum. 2004. "An Elevated Basal FSH Reflects a Quantitative Rather than Qualitative Decline of the Ovarian Reserve." *Human Reproduction (Oxford, England)* 19 (4):893–98. https://doi.org/10.1093/humrep/deh141.

Abdelbaki, Shabieb A., Jehan H. Sabry, Ahmed M. Al-Adl, and Hanan H. Sabry. 2017. "The Impact of Coexisting Sperm DNA Fragmentation and Seminal Oxidative Stress on the Outcome of Varicocelectomy in Infertile Patients: A Prospective Controlled Study." *Arab Journal of Urology* 15 (2):131–39. https://doi.org/10.1016/j.aju.2017.03.002.

Acharya, Ganesh, Sven-Erik Sonesson, Kari Flo, Juha Räsänen, and Anthony Odibo. 2016. "Hemodynamic Aspects of Normal Human Feto-Placental (Umbilical) Circulation." *Acta Obstetricia Et Gynecologica Scandinavica* 95 (6):672–82. https://doi.org/10.1111/aogs.12919.

Adams, David R., Murat Sincan, Karin Fuentes Fajardo, James C. Mullikin, Tyler M. Pierson, Camilo Toro, Cornelius F. Boerkoel, Cynthia J. Tifft, William A. Gahl, and Tom C. Markello. 2012. "Analysis of DNA Sequence Variants Detected by High-Throughput Sequencing." *Human Mutation* 33 (4):599–608. https://doi.org/10.1002/humu.22035.

Adler, Alexis, Hsaio-Ling Lee, David H. McCulloh, Esmeralda Ampeloquio, Melicia Clarke-Williams, Brooke Hodes Wertz, and James Grifo. 2014. "Blastocyst Culture Selects for Euploid Embryos: Comparison of Blastomere and Trophectoderm Biopsies." *Reproductive Biomedicine Online* 28 (4):485–91. https://doi.org/10.1016/j.rbmo.2013.11.018.

Aghajanova, Lusine, Jacquelyn Hoffman, Evelyn Mok-Lin, and Christopher N. Herndon. 2016. "Obstetrics and Gynecology Residency and Fertility Needs: National Survey Results." *Reproductive Sciences (Thousand Oaks, Calif.)*, June. https://doi.org/10.1177/1933719116657193.

Agrawal, Anurag A. 2001. "Phenotypic Plasticity in the Interactions and Evolution of Species." *Science* 294 (5541):321–26. https://doi.org/10.1126/science.1060701.

Ahmad, Ausaf, and M. Anwar Iqbal. 2012. "Significance of Genome-Wide Analysis of Copy Number Alterations and UPD in Myelodysplastic Syndromes Using Combined CGH - SNP Arrays." *Current Medicinal Chemistry* 19 (22):3739–47.

Alborzi, S., G. Madadi, A. Samsami, P. Soheil, M. Azizi, M. Alborzi, and P. Bakhshaie. 2015. "Decreased Ovarian Reserve: Any New Hope?" *Minerva Ginecologica* 67 (2):149–67.

Aleksandrova, N., E. Shubina, A. Ekimov, T. Kodyleva, I. Mukosey, N. Makarova, E. Kulakova, L. Levkov, D. Trofimov, and G. Sukhikh. 2016. "Comparison of the Results of Preimplantation Genetic Screening Obtained by A-CGH and NGS Methods from the Same Embryos." *Gynecological Endocrinology: The Official Journal of the International Society of Gynecological Endocrinology* 32 (sup2):1–4. https://doi.org/10.1080/09513590.2016.1232892.

Alfarawati, Samer, Elpida Fragouli, Pere Colls, John Stevens, Cristina Gutiérrez-Mateo, William B. Schoolcraft, Mandy G. Katz-Jaffe, and Dagan Wells. 2011. "The Relationship between Blastocyst Morphology, Chromosomal Abnormality, and Embryo Gender." *Fertility and Sterility* 95 (2):520–24. https://doi.org/10.1016/j.fertnstert.2010.04.003.

Ali, Salah, Anna Gacsadi, Elizabeth McDougall, Christine Armstrong, Joerg Krueger, Tal Schechter, and Muhammad Ali. 2017. "Successful Reduced Intensity Allogeneic Transplant With Full Donor Chimerism and Good Quality of Life in Adolescent Patient With Wiskott-Aldrich Syndrome." *Journal of Pediatric Hematology/Oncology* 39 (5):e267–69. https://doi.org/10.1097/MPH.0000000000000851.

Alikani, Mina. 2005. "Epithelial Cadherin Distribution in Abnormal Human Pre-Implantation Embryos." *Human Reproduction* 20 (12):3369–75. https://doi.org/10.1093/humrep/dei242.

Alvarez, Silvia. 2015. "Do Some Addictions Interfere with Fertility?" *Fertility and Sterility* 103 (1):22–26. https://doi.org/10.1016/j.fertnstert.2014.11.008.

AN, Li-You, Sanjeev A CHAUBAL, Yanhong LIU, Yexiang CHEN, Tshimangadzo L NEDAMBALE, Jie XU, Fei XUE, et al. 2017. "Significant Heparin Effect on Bovine Embryo Development during Sexed in Vitro Fertilization." *The Journal of Reproduction and Development* 63 (2):175–83. https://doi.org/10.1262/jrd.2016-142.

Andersen, C. Yding, R. Fischer, V. Giorgione, and Thomas W. Kelsey. 2016. "Micro-Dose HCG as Luteal Phase Support without Exogenous Progesterone Administration: Mathematical Modelling of the HCG Concentration in Circulation and Initial Clinical Experience." *Journal of Assisted Reproduction and Genetics*, July. https://doi.org/10.1007/s10815-016-0764-7.

Archibald, F. 2003. "Oxygen Toxicity and the Health and Survival of Eukaryote Cells: A New Piece Is Added to the Puzzle." *Proceedings of the National Academy of Sciences of the United States of America* 100 (18):10141–43. https://doi.org/10.1073/pnas.1934513100.

Atasever, Melahat, Zeynep Soyman, Emine Demirel, Servet Gencdal, and Sefa Kelekci. 2016. "Diminished Ovarian Reserve: Is It a Neglected Cause for Assessment Recurrent Miscarriage? A Cohort Study." *Fertility and Sterility*, January. https://doi.org/10.1016/j.fertnstert.2016.01.001.

Atlas de Reproducción Asistida. 2014. *EMBRYO DEVELOPMENT AND IMPLANTATION*. https://www.youtube.com/watch?v=3u251OXfsRU.

Babb, S. G., J. Barnett, A. L. Doedens, N. Cobb, Q. Liu, B. C. Sorkin, P. C. Yelick, P. A. Raymond, and J. A. Marrs. 2001. "Zebrafish E-Cadherin: Expression during Early Embryogenesis and Regulation during Brain Development." *Developmental Dynamics: An Official Publication of the American Association of Anatomists* 221 (2):231–37. https://doi.org/10.1002/dvdy.1132.

Baltz, Jay M. 2012. "Media Composition: Salts and Osmolality." *Methods in Molecular Biology (Clifton, N.J.)* 912:61–80. https://doi.org/10.1007/978-1-61779-971-6_5.

Banjanovic, Erin S., and Jason W. Osborne. 2016. "Confidence Intervals for Effect Sizes: Applying Bootstrap Resampling." *Practical Assessment, Research & Evaluation* 21.

Barnett, D. K., and B. D. Bavister. 1996. "What Is the Relationship between the Metabolism of Preimplantation Embryos and Their Developmental Competence?" *Molecular Reproduction and Development* 43 (1):105–33. https://doi.org/10.1002/(SICI)1098-2795(199601)43:1<105::AID-MRD13>3.0.CO;2-4.

Barroso, Gerardo, Sergio Oehninger, Ana Monzó, Paul Kolm, William E. Gibbons, and Suheil J. Muasher. 2001. "Clinical Assisted Reproduction: High FSH:LH Ratio and Low LH Levels in Basal Cycle Day 3: Impact on Follicular Development and IVF Outcome." *Journal of Assisted Reproduction and Genetics* 18 (9):499–505. https://doi.org/10.1023/A:1016601110424.

Basilicata, M. Felicia, Marcus Frank, Davor Solter, Thomas Brabletz, and Marc P. Stemmler. 2016. "Inappropriate Cadherin Switching in the Mouse Epiblast Compromises Proper Signaling between the Epiblast and the Extraembryonic Ectoderm during Gastrulation." *Scientific Reports* 6:26562. https://doi.org/10.1038/srep26562.

Basson, M. Albert. 2012. "Signaling in Cell Differentiation and Morphogenesis." *Cold Spring Harbor Perspectives in Biology* 4 (6). https://doi.org/10.1101/cshperspect.a008151.

Baştanlar, Yalin, and Mustafa Ozuysal. 2014. "Introduction to Machine Learning." *Methods in Molecular Biology (Clifton, N.J.)* 1107:105–28. https://doi.org/10.1007/978-1-62703-748-8_7.

Bauer, Bethany K., S. Clay Isom, Lee D. Spate, Kristin M. Whitworth, William G. Spollen, Sean M. Blake, Gordon K. Springer, Clifton N. Murphy, and Randall S. Prather. 2010. "Transcriptional Profiling by Deep Sequencing Identifies Differences in MRNA Transcript Abundance in in Vivo-Derived versus in Vitro-Cultured Porcine Blastocyst Stage Embryos." *Biology of Reproduction* 83 (5):791–98. https://doi.org/10.1095/biolreprod.110.085936.

Bavister, Barry D. 2012. *The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation in Vitro*. Springer Science & Business Media.

Beal, Marc A., Carole L. Yauk, and Francesco Marchetti. 2017. “From Sperm to Offspring: Assessing the Heritable Genetic Consequences of Paternal Smoking and Potential Public Health Impacts.” *Mutation Research* 773 (July):26–50. https://doi.org/10.1016/j.mrrev.2017.04.001.

Bedaiwy, Mohamed A., Tommaso Falcone, Maher S. Mohamed, Abdel A. N. Aleem, Rakesh K. Sharma, Sarah E. Worley, Julie Thornton, and Ashok Agarwal. 2004. “Differential Growth of Human Embryos in Vitro: Role of Reactive Oxygen Species.” *Fertility and Sterility* 82 (3):593–600. https://doi.org/10.1016/j.fertnstert.2004.02.121.

Bedzhov, Ivan, Sarah J. L. Graham, Chuen Yan Leung, and Magdalena Zernicka-Goetz. 2014. “Developmental Plasticity, Cell Fate Specification and Morphogenesis in the Early Mouse Embryo.” *Philosophical Transactions of the Royal Society B: Biological Sciences* 369 (1657). https://doi.org/10.1098/rstb.2013.0538.

Belva, F., M. Bonduelle, M. Roelants, D. Michielsen, A. Van Steirteghem, G. Verheyen, and H. Tournaye. 2016. “Semen Quality of Young Adult ICSI Offspring: The First Results.” *Human Reproduction (Oxford, England)*, October. https://doi.org/10.1093/humrep/dew245.

Benirschke, K., F. Naftolin, R. Gittes, G. Khudr, S. S. Yen, and F. H. Allen. 1972. “True Hermaphroditism and Chimerism. A Case Report.” *American Journal of Obstetrics and Gynecology* 113 (4):449–58.

Ben-Rafael, Z., and D. Feldberg. 1993. “The Poor-Responder Patient in an in Vitro Fertilization-Embryo Transfer Program.” *Journal of Assisted Reproduction and Genetics* 10 (2):118–20.

Bens, Susanne, Manuel Luedeke, Tanja Richter, Melanie Graf, Julia Kolarova, Gotthold Barbi, Krisztian Lato, Thomas F. Barth, and Reiner Siebert. 2017. “Mosaic Genome-Wide Maternal Isodiploidy: An Extreme Form of Imprinting Disorder Presenting as Prenatal Diagnostic Challenge.” *Clinical Epigenetics* 9:111. https://doi.org/10.1186/s13148-017-0410-y.

Breau, Marie Anne, and Sylvie Schneider-Maunoury. 2015. “Cranial Placodes: Models for Exploring the Multi-Facets of Cell Adhesion in Epithelial Rearrangement, Collective Migration and Neuronal Movements.” *Developmental Biology* 401 (1):25–36. https://doi.org/10.1016/j.ydbio.2014.12.012.

Breiman, Leo. 2001. “Random Forests.” *Machine Learning* 45 (1):5–32. https://doi.org/10.1023/A:1010933404324.

Brodland, G. Wayne. 2015. “How Computational Models Can Help Unlock Biological Systems.” *Seminars in Cell & Developmental Biology*, Coding and non-coding RNAs & Mammalian development, 47–48 (Supplement C):62–73. https://doi.org/10.1016/j.semcdb.2015.07.001.

Broekmans, F. J., M. R. Soules, and B. C. Fauser. 2009. “Ovarian Aging: Mechanisms and Clinical Consequences.” *Endocrine Reviews* 30 (5):465–93. https://doi.org/10.1210/er.2009-0006.

Broer, S. L., M. Dólleman, B. C. Opmeer, B. C. Fauser, B. W. Mol, and F. J. M. Broekmans. 2011. “AMH and AFC as Predictors of Excessive Response in Controlled Ovarian Hyperstimulation: A Meta-Analysis.” *Human Reproduction Update* 17 (1):46–54. https://doi.org/10.1093/humupd/dmq034.

Broer, Simone L., Frank J. M. Broekmans, Joop S. E. Laven, and Bart C. J. M. Fauser. 2014. “Anti-Müllerian Hormone: Ovarian Reserve Testing and Its Potential Clinical Implications.” *Human Reproduction Update* 20 (5):688–701. https://doi.org/10.1093/humupd/dmu020.

Bronner, F. 2001. “Extracellular and Intracellular Regulation of Calcium Homeostasis.” *TheScientificWorldJournal* 1 (December):919–25. https://doi.org/10.1100/tsw.2001.489.

Bui, Masato, Beben Benyamin, Sonia Shah, Anjali K. Henders, Nicholas G. Martin, Grant W. Montgomery, and Allan F. McRae. 2015. “Sharing a Placenta Is Associated With a Greater Similarity in DNA Methylation in Monochorionic Versus Dichorionic Twin Pars in Blood at Age 14.” *Twin Research and Human Genetics: The Official Journal of the International Society for Twin Studies* 18 (6):680–85. https://doi.org/10.1017/thg.2015.87.

Bungum, M., P. Humaidan, and L. Bungum. 2002. “Recombinant Human Albumin as Protein Source in Culture Media Used for IVF: A Prospective Randomized Study.” *Reproductive Biomedicine Online* 4 (3):233–36.

Butts, Samantha F., Sarah Ratcliffe, Anuja Dokras, and David B. Seifer. 2013. “Diagnosis and Treatment of Diminished Ovarian Reserve in Assisted Reproductive Technology Cycles of Women up to Age 40 Years: The Role of Insurance Mandates.” *Fertility and Sterility* 99 (2):382–88. https://doi.org/10.1016/j.fertnstert.2012.09.026.

Callaci, Sandhya, Kylee Morrison, Xiangqiang Shao, Amber L. Schuh, Yueju Wang, John R. Yates, Jeff Hardin, and Anjon Audhya. 2015. “Phosphoregulation of the C. Elegans Cadherin-Catenin Complex.” *The Biochemical Journal* 472 (3):339–52. https://doi.org/10.1042/BJ20150410.

Câmara, D. R., J. P. Kastelic, and J. C. Thundathil. 2016. “Role of the Na+/K+-ATPase Ion Pump in Male Reproduction and Embryo Development.” *Reproduction, Fertility, and Development*, July. https://doi.org/10.1071/RD16091.

Canty, Angelo, and Brian Ripley. 2017. *Package ‘Boot.’* Version.

Capalbo, Antonio, Laura Rienzi, Danilo Cimadomo, Roberta Maggiulli, Thomas Elliott, Graham Wright, Zsolt Peter Nagy, and Filippo Maria Ubaldi. 2014. “Correlation between Standard Blastocyst Morphology, Euploidy and Implantation: An Observational Study in

Two Centers Involving 956 Screened Blastocysts." *Human Reproduction (Oxford, England)* 29 (6):1173–81. https://doi.org/10.1093/humrep/deu033.

Capalbo, Antonio, Filippo Maria Ubaldi, Laura Rienzi, Richard Scott, and Nathan Treff. 2017. "Detecting Mosaicism in Trophectoderm Biopsies: Current Challenges and Future Possibilities." *Human Reproduction (Oxford, England)* 32 (3):492–98. https://doi.org/10.1093/humrep/dew250.

Carter, A. M., and A. C. Enders. 2016. "Placentation in Mammals: Definitive Placenta, Yolk Sac, and Paraplacenta." *Theriogenology* 86 (1):278–87. https://doi.org/10.1016/j.theriogenology.2016.04.041.

Chapman, Chevy, Lynsey Cree, and Andrew N Shelling. 2015. "The Genetics of Premature Ovarian Failure: Current Perspectives." *International Journal of Women's Health* 7 (September):799–810. https://doi.org/10.2147/IJWH.S64024.

Cheatham, Mary Ann, Sharon Low-Zeddies, Khurram Naik, Roxanne Edge, Jing Zheng, Charles T. Anderson, and Peter Dallos. 2009. "A Chimera Analysis of Prestin Knock-out Mice." *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 29 (38):12000–8. https://doi.org/10.1523/JNEUROSCI.1651-09.2009.

Chitwood, James L., Victoria R. Burruel, Michelle M. Halstead, Stuart A. Meyers, and Pablo J. Ross. 2017. "Transcriptome Profiling of Individual Rhesus Macaque Oocytes and Preimplantation Embryos." *Biology of Reproduction* 97 (3):353–64. https://doi.org/10.1093/biolre/iox114.

Cochran, David M., Dai Fukumura, Marek Ancukiewicz, Peter Carmeliet, and Rakesh K. Jain. 2006. "Evolution of Oxygen and Glucose Concentration Profiles in a Tissue-Mimetic Culture System of Embryonic Stem Cells." *Annals of Biomedical Engineering* 34 (8):1247–58. https://doi.org/10.1007/s10439-006-9134-4.

Cohen, J., N. Chabbert-Buffet, and E. Darai. 2015. "Diminished Ovarian Reserve, Premature Ovarian Failure, Poor Ovarian Responder-a Plea for Universal Definitions." *Journal of Assisted Reproduction and Genetics* 32 (12):1709–12. https://doi.org/10.1007/s10815-015-0595-y.

Corcoran, Jemma J., Christopher Nicholson, Michèle Sweeney, Jayne C. Charnock, Stephen C. Robson, Melissa Westwood, and Michael J. Taggart. 2014. "Human Uterine and Placental Arteries Exhibit Tissue-Specific Acute Responses to 17β-Estradiol and Estrogen-Receptor-Specific Agonists." *Molecular Human Reproduction* 20 (5):433–41. https://doi.org/10.1093/molehr/gat095.

Costa-Borges, Nuno, Marta Bellés, Marcos Meseguer, Daniela Galliano, Agustin Ballesteros, and Gloria Calderón. 2016. "Blastocyst Development in Single Medium with or without Renewal on Day 3: A Prospective Cohort Study on Sibling Donor Oocytes in a Time-Lapse Incubator." *Fertility and Sterility* 105 (3):707–13. https://doi.org/10.1016/j.fertnstert.2015.11.038.

Dahdouh, Elias M., Jacques Balayla, François Audibert, Genetics Committee, R. Douglas Wilson, François Audibert, Jo-Ann Brock, et al. 2015. “Technical Update: Preimplantation Genetic Diagnosis and Screening.” *Journal of Obstetrics and Gynaecology Canada: JOGC = Journal d’obstétrique et Gynécologie Du Canada: JOGC* 37 (5):451–63.

Daughtry, Brittany L., and Shawn L. Chavez. 2016. “Chromosomal Instability in Mammalian Pre-Implantation Embryos: Potential Causes, Detection Methods, and Clinical Consequences.” *Cell and Tissue Research* 363 (1):201–25. https://doi.org/10.1007/s00441-015-2305-6.

Davison, Anthony Christopher, and David Victor Hinkley. 1997. *Bootstrap Methods and Their Application*. Vol. 1. Cambridge university press.

De Jonge, Christopher J. 2017. “A Clinical Assay for Reactive Oxygen Species - Ready for Primetime?” *Reproductive Biomedicine Online*, October. https://doi.org/10.1016/j.rbmo.2017.09.006.

Delhanty, J. D., D. K. Griffin, A. H. Handyside, J. Harper, G. H. Atkinson, M. H. Pieters, and R. M. Winston. 1993. “Detection of Aneuploidy and Chromosomal Mosaicism in Human Embryos during Preimplantation Sex Determination by Fluorescent in Situ Hybridisation, (FISH).” *Human Molecular Genetics* 2 (8):1183–85.

Denomme, Michelle M., Blair R. McCallie, Jason C. Parks, William B. Schoolcraft, and Mandy G. Katz-Jaffe. 2017. “Alterations in the Sperm Histone-Retained Epigenome Are Associated with Unexplained Male Factor Infertility and Poor Blastocyst Development in Donor Oocyte IVF Cycles.” *Human Reproduction (Oxford, England)*, October, 1–13. https://doi.org/10.1093/humrep/dex317.

Denver, Robert J., and Erica J. Crespi. 2006. “Stress Hormones and Human Developmental Plasticity.” *NeoReviews* 7 (4):e183–e188.

Deo, Rahul C. 2015. “Machine Learning in Medicine.” *Circulation* 132 (20):1920–30. https://doi.org/10.1161/CIRCULATIONAHA.115.001593.

Dewailly, Didier, Claus Yding Andersen, Adam Balen, Frank Broekmans, Nafi Dilaver, Renato Fanchin, Georg Griesinger, et al. 2014. “The Physiology and Clinical Utility of Anti-Mullerian Hormone in Women.” *Human Reproduction Update* 20 (3):370–85. https://doi.org/10.1093/humupd/dmt062.

Dubyak, George R. 2004. “Ion Homeostasis, Channels, and Transporters: An Update on Cellular Mechanisms.” *Advances in Physiology Education* 28 (1–4):143–54. https://doi.org/10.1152/advan.00046.2004.

Dumoulin, J. C., C. J. Meijers, M. Bras, E. Coonen, J. P. Geraedts, and J. L. Evers. 1999. “Effect of Oxygen Concentration on Human In-Vitro Fertilization and Embryo Culture.” *Human Reproduction (Oxford, England)* 14 (2):465–69.

Durlinger, A. L., P. Kramer, B. Karels, F. H. de Jong, J. T. Uilenbroek, J. A. Grootegoed, and A. P. Themmen. 1999. “Control of Primordial Follicle Recruitment by Anti-Müllerian Hormone in the Mouse Ovary.” *Endocrinology* 140 (12):5789–96. https://doi.org/10.1210/endo.140.12.7204.

Durlinger, Alexandra L. L., Maria J. G. Gruijters, Piet Kramer, Bas Karels, Holly A. Ingraham, Mark W. Nachtigal, Jan Th J. Uilenbroek, J. Anton Grootegoed, and Axel P. N. Themmen. 2002. “Anti-Müllerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary.” *Endocrinology* 143 (3):1076–84. https://doi.org/10.1210/endo.143.3.8691.

Edelman, Lucas B., Sriram Chandrasekaran, and Nathan D. Price. 2010. “Systems Biology of Embryogenesis.” *Reproduction, Fertility, and Development* 22 (1):98–105. https://doi.org/10.1071/RD09215.

Eiben, A.E., and J.E. Smith. 2007. *Introduction to Evolutionary Computing*. New York, NY: Springer-Verlag. //www.springer.com/us/book/9783642072857.

Epstein, C. J., and S. A. Smith. 1973. “Amino Acid Uptake and Protein Synthesis in Preimplanatation Mouse Embryos.” *Developmental Biology* 33 (1):171–84.

Esposito, M. A., C. Coutifaris, and K. T. Barnhart. 2002. “A Moderately Elevated Day 3 FSH Concentration Has Limited Predictive Value, Especially in Younger Women.” *Human Reproduction (Oxford, England)* 17 (1):118–23.

Ethics Committee of American Society for Reproductive Medicine. 2012. “Fertility Treatment When the Prognosis Is Very Poor or Futile: A Committee Opinion.” *Fertility and Sterility* 98 (1):e6-9. https://doi.org/10.1016/j.fertnstert.2012.03.045.

Evans, Jemma, Lois A. Salamonsen, Amy Winship, Ellen Menkhorst, Guiying Nie, Caroline E. Gargett, and Eva Dimitriadis. 2016. “Fertile Ground: Human Endometrial Programming and Lessons in Health and Disease.” *Nature Reviews. Endocrinology*, July. https://doi.org/10.1038/nrendo.2016.116.

Fan, G, and Gray, JB. 2005. “Regression Tree Analysis Using TARGET” 14 (1):206–18.

Farquhar, Cindy, Josephine R Rishworth, Julie Brown, Willianne LDM Nelen, and Jane Marjoribanks. 2013. “Assisted Reproductive Technology: An Overview of Cochrane Reviews.” In *Cochrane Database of Systematic Reviews*. John Wiley & Sons, Ltd. http://onlinelibrary.wiley.com/doi/10.1002/14651858.CD010537.pub2/abstract.

“FastStats.” 2017. National Center for Health Statistics. July 5, 2017. https://www.cdc.gov/nchs/fastats/infertility.htm.

Fawzy, Mohamed, Mai Emad, Mohamed Y. AbdelRahman, Hazem Abdelghafar, Faten F. Abdel Hafez, and Mohamed A. Bedaiwy. 2017. “Impact of 3.5% O2 Culture on Embryo Development and Clinical Outcomes: A Comparative Study.” *Fertility and Sterility*, August. https://doi.org/10.1016/j.fertnstert.2017.07.013.

Feuer, Sky K., Annemarie Donjacour, Rhodel K. Simbulan, Wingka Lin, Xiaowei Liu, Emin Maltepe, and Paolo F. Rinaudo. 2014. “Sexually Dimorphic Effect of In Vitro Fertilization (IVF) on Adult Mouse Fat and Liver Metabolomes.” *Endocrinology* 155 (11):4554–67. https://doi.org/10.1210/en.2014-1465.

Fiorentino, Francesco, Anil Biricik, Sara Bono, Letizia Spizzichino, Ettore Cotroneo, Giuliano Cottone, Felix Kokocinski, and Claude-Edouard Michel. 2014. “Development and Validation of a Next-Generation Sequencing-Based Protocol for 24-Chromosome Aneuploidy Screening of Embryos.” *Fertility and Sterility* 101 (5):1375–82. https://doi.org/10.1016/j.fertnstert.2014.01.051.

Fiorentino, Francesco, Sara Bono, Anil Biricik, Andrea Nuccitelli, Ettore Cotroneo, Giuliano Cottone, Felix Kokocinski, Claude-Edouard Michel, Maria Giulia Minasi, and Ermanno Greco. 2014. “Application of Next-Generation Sequencing Technology for Comprehensive Aneuploidy Screening of Blastocysts in Clinical Preimplantation Genetic Screening Cycles.” *Human Reproduction (Oxford, England)* 29 (12):2802–13. https://doi.org/10.1093/humrep/deu277.

Fischer, B., and B. D. Bavister. 1993. “Oxygen Tension in the Oviduct and Uterus of Rhesus Monkeys, Hamsters and Rabbits.” *Journal of Reproduction and Fertility* 99 (2):673–79.

Fleming, T. P., M. R. Ghassemifar, and B. Sheth. 2000. “Junctional Complexes in the Early Mammalian Embryo.” *Seminars in Reproductive Medicine* 18 (2):185–93. https://doi.org/10.1055/s-2000-12557.

Fleming, T. P., B. Sheth, and I. Fesenko. 2001. “Cell Adhesion in the Preimplantation Mammalian Embryo and Its Role in Trophectoderm Differentiation and Blastocyst Morphogenesis.” *Frontiers in Bioscience: A Journal and Virtual Library* 6 (August):D1000-1007.

Fragouli, Elpida, and Dagan Wells. 2012. “Aneuploidy Screening for Embryo Selection.” *Seminars in Reproductive Medicine* 30 (4):289–301. https://doi.org/10.1055/s-0032-1313908.

Froud, Robert, and Gary Abel. 2014. “Using ROC Curves to Choose Minimally Important Change Thresholds When Sensitivity and Specificity Are Valued Equally: The Forgotten Lesson of Pythagoras. Theoretical Considerations and an Example Application of Change in Health Status.” *PloS One* 9 (12):e114468. https://doi.org/10.1371/journal.pone.0114468.

Gardner, David K. 1998. “Changes in Requirements and Utilization of Nutrients during Mammalian Preimplantation Embryo Development and Their Significance in Embryo Culture.” *Theriogenology* 49 (1):83–102.

Gardner, David K., R. Hamilton, B. McCallie, W. B. Schoolcraft, and M. G. Katz-Jaffe. 2013. “Human and Mouse Embryonic Development, Metabolism and Gene Expression Are Altered by an Ammonium Gradient in Vitro.” *Reproduction (Cambridge, England)* 146 (1):49–61. https://doi.org/10.1530/REP-12-0348.

Gardner, David K., and Alexandra J. Harvey. 2015. “Blastocyst Metabolism.” *Reproduction, Fertility, and Development* 27 (4):638–54. https://doi.org/10.1071/RD14421.

Gardner, David K., and R. L. Kelley. 2017. “Impact of the IVF Laboratory Environment on Human Preimplantation Embryo Phenotype.” *Journal of Developmental Origins of Health and Disease* 8 (4):418–35. https://doi.org/10.1017/S2040174417000368.

Gardner, David K., and M. Lane. 1997. “Culture and Selection of Viable Blastocysts: A Feasible Proposition for Human IVF?” *Human Reproduction Update* 3 (4):367–82.

Gardner, David K., M. Lane, A. Spitzer, and P. A. Batt. 1994. “Enhanced Rates of Cleavage and Development for Sheep Zygotes Cultured to the Blastocyst Stage in Vitro in the Absence of Serum and Somatic Cells: Amino Acids, Vitamins, and Culturing Embryos in Groups Stimulate Development.” *Biology of Reproduction* 50 (2):390–400.

Gardner, David K., and H. J. Leese. 1990. “Concentrations of Nutrients in Mouse Oviduct Fluid and Their Effects on Embryo Development and Metabolism in Vitro.” *Journal of Reproduction and Fertility* 88 (1):361–68.

Gardner, David K., Laura Reed, Donald Linck, Courtney Sheehan, and Michelle Lane. 2005. “Quality Control in Human in Vitro Fertilization.” *Seminars in Reproductive Medicine* 23 (4):319–24. https://doi.org/10.1055/s-2005-923389.

Gardner, David K., and Carlos Simón. 2017. *Handbook of In Vitro Fertilization, Fourth Edition*. CRC Press.

Gardner, David K., and Petra L. Wale. 2013. “Analysis of Metabolism to Select Viable Human Embryos for Transfer.” *Fertility and Sterility* 99 (4):1062–72. https://doi.org/10.1016/j.fertnstert.2012.12.004.

Gielen, Marij, Patrick J. Lindsey, Catherine Derom, Ruth J. F. Loos, Robert Derom, Jan G. Nijhuis, and Robert Vlietinck. 2006. “Curves of Placental Weights of Live-Born Twins.” *Twin Research and Human Genetics: The Official Journal of the International Society for Twin Studies* 9 (5):664–72. https://doi.org/10.1375/183242706778553471.

Gilbert, Scott F. 2000. “Diachronic Biology Meets Evo-Devo: C. H. Waddington’s Approach to Evolutionary Developmental Biology.” *Integrative and Comparative Biology* 40 (5):729–37. https://doi.org/10.1093/icb/40.5.729.

———. 2014. *Developmental Biology*. Tenth edition. Sunderland, MA, USA: Sinauer Associates, Inc., Publishers.

Gill, Sabrina, Julie L. Sharpless, Kimberly Rado, and Janet E. Hall. 2002. “Evidence That GnRH Decreases with Gonadal Steroid Feedback but Increases with Age in Postmenopausal Women.” *The Journal of Clinical Endocrinology & Metabolism* 87 (5):2290–96. https://doi.org/10.1210/jcem.87.5.8508.

Giritharan, G., L. Delle Piane, A. Donjacour, F. J. Esteban, J. A. Horcajadas, E. Maltepe, and P. Rinaudo. 2012. “In Vitro Culture of Mouse Embryos Reduces Differential Gene Expression Between Inner Cell Mass and Trophectoderm.” *Reproductive Sciences* 19 (3):243–52. https://doi.org/10.1177/1933719111428522.

Gleicher, Norbert, Vitaly A Kushnir, and David H Barad. 2014. “Preimplantation Genetic Screening (PGS) Still in Search of a Clinical Application: A Systematic Review.” *Reproductive Biology and Endocrinology : RB&E* 12 (March):22. https://doi.org/10.1186/1477-7827-12-22.

Gleicher, Norbert, Jacob Metzger, Gist Croft, Vitaly A. Kushnir, David F. Albertini, and David H. Barad. 2017. “A Single Trophectoderm Biopsy at Blastocyst Stage Is Mathematically Unable to Determine Embryo Ploidy Accurately Enough for Clinical Use.” *Reproductive Biology and Endocrinology: RB&E* 15 (1):33. https://doi.org/10.1186/s12958-017-0251-8.

Gleicher, Norbert, and Raoul Orvieto. 2017. “Is the Hypothesis of Preimplantation Genetic Screening (PGS) Still Supportable? A Review.” *Journal of Ovarian Research* 10 (1):21. https://doi.org/10.1186/s13048-017-0318-3.

Gnoth, C., D. Godehardt, E. Godehardt, P. Frank-Herrmann, and G. Freundl. 2003. “Time to Pregnancy: Results of the German Prospective Study and Impact on the Management of Infertility.” *Human Reproduction* 18 (9):1959–66. https://doi.org/10.1093/humrep/deg366.

Goodwin, Sara, John D. McPherson, and W. Richard McCombie. 2016. “Coming of Age: Ten Years of next-Generation Sequencing Technologies.” *Nature Reviews Genetics* 17 (6):333–51. https://doi.org/10.1038/nrg.2016.49.

Gray, Henry. 2000. *Anatomy of the Human Body*. 20th ed. Philadelphia: Lea & Febiger.

Greco, Ermanno, Sara Bono, Alessandra Ruberti, Anna Maria Lobascio, Pierfrancesco Greco, Anil Biricik, Letizia Spizzichino, et al. 2014. “Comparative Genomic Hybridization Selection of Blastocysts for Repeated Implantation Failure Treatment: A Pilot Study.” *BioMed Research International* 2014. https://doi.org/10.1155/2014/457913.

Gromski, Piotr S., Yun Xu, Elon Correa, David I. Ellis, Michael L. Turner, and Royston Goodacre. 2014. “A Comparative Investigation of Modern Feature Selection and Classification Approaches for the Analysis of Mass Spectrometry Data.” *Analytica Chimica Acta* 829 (June):1–8. https://doi.org/10.1016/j.aca.2014.03.039.

Grubinger, T., Achim, Pfeifer, and Karl-Peter. 2014. “Evtree: Evolutionary Learning of Globally Optimal Classifcation and Regression Trees in R.” *Journal of Statistical Software* 61 (1):29.

Grynnerup, Anna Garcia–Alix, Anette Lindhard, and Steen Sørensen. 2012. “The Role of Anti-Müllerian Hormone in Female Fertility and Infertility – an Overview.” *Acta Obstetricia*

*et Gynecologica Scandinavica* 91 (11):1252–60. https://doi.org/10.1111/j.1600-0412.2012.01471.x.

Guérin, P., S. El Mouatassim, and Y. Ménézo. 2001. "Oxidative Stress and Protection against Reactive Oxygen Species in the Pre-Implantation Embryo and Its Surroundings." *Human Reproduction Update* 7 (2):175–89.

Guo, Na, Yufeng Li, Jihui Ai, Longjie Gu, Wen Chen, and Qun Liu. 2014. "Two Different Concentrations of Oxygen for Culturing Precompaction Stage Embryos on Human Embryo Development Competence: A Prospective Randomized Sibling-Oocyte Study." *International Journal of Clinical and Experimental Pathology* 7 (9):6191–98.

Gwatkin, Ralph B. L. 1993. "Handbook of in Vitro Fertilization, Edited by Alan O. Trounson and David K. Gardner, CRC Press, Boca Raton, FL, 1993, 324 Pp, $89.50." *Molecular Reproduction and Development* 36 (4):517–517. https://doi.org/10.1002/mrd.1080360418.

Hajian-Tilaki, Karimollah. 2013. "Receiver Operating Characteristic (ROC) Curve Analysis for Medical Diagnostic Test Evaluation." *Caspian Journal of Internal Medicine* 4 (2):627–35.

Hallam, Stephanie, Heather Nelson, Valerie Greger, Cynthia Perreault-Micale, Jocelyn Davie, Nicole Faulkner, Dana Neitzel, et al. 2014. "Validation for Clinical Use of, and Initial Clinical Experience with, a Novel Approach to Population-Based Carrier Screening Using High-Throughput, Next-Generation DNA Sequencing." *The Journal of Molecular Diagnostics* 16 (2):180–89. https://doi.org/10.1016/j.jmoldx.2013.10.006.

Handwerger, S., and M. Freemark. 2000. "The Roles of Placental Growth Hormone and Placental Lactogen in the Regulation of Human Fetal Growth and Development." *Journal of Pediatric Endocrinology & Metabolism: JPEM* 13 (4):343–56.

Handyside, Alan H. 2013. "24-Chromosome Copy Number Analysis: A Comparison of Available Technologies." *Fertility and Sterility* 100 (3):595–602. https://doi.org/10.1016/j.fertnstert.2013.07.1965.

Handyside, Alan H., Markus Montag, M. Cristina Magli, Sjoerd Repping, Joyce Harper, Andreas Schmutzler, Katerina Vesela, Luca Gianaroli, and Joep Geraedts. 2012. "Multiple Meiotic Errors Caused by Predivision of Chromatids in Women of Advanced Maternal Age Undergoing in Vitro Fertilisation." *European Journal of Human Genetics: EJHG* 20 (7):742–47. https://doi.org/10.1038/ejhg.2011.272.

Hardy, K., A. Warner, R. M. Winston, and D. L. Becker. 1996. "Expression of Intercellular Junctions during Preimplantation Development of the Human Embryo." *Molecular Human Reproduction* 2 (8):621–32.

Hariton, Eduardo, Keewan Kim, Sunni L. Mumford, Marissa Palmor, Pietro Bortoletto, Eden R. Cardozo, Anatte E. Karmon, Mary E. Sabatini, and Aaron K. Styer. 2017. "Total Number of Oocytes and Zygotes Are Predictive of Live Birth Pregnancy in Fresh Donor Oocyte

in Vitro Fertilization Cycles." *Fertility and Sterility* 108 (2):262–68. https://doi.org/10.1016/j.fertnstert.2017.05.021.

Harper, Joyce C., and Gary Harton. 2010. "The Use of Arrays in Preimplantation Genetic Diagnosis and Screening." *Fertility and Sterility* 94 (4):1173–77. https://doi.org/10.1016/j.fertnstert.2010.04.064.

Harper, Joyce, Edith Coonen, Martine De Rycke, Francesco Fiorentino, Joep Geraedts, Veerle Goossens, Gary Harton, et al. 2010. "What next for Preimplantation Genetic Screening (PGS)? A Position Statement from the ESHRE PGD Consortium Steering Committee." *Human Reproduction (Oxford, England)* 25 (4):821–23. https://doi.org/10.1093/humrep/dep476.

Harris, Tony J. C., and Ulrich Tepass. 2010. "Adherens Junctions: From Molecules to Morphogenesis." *Nature Reviews Molecular Cell Biology* 11 (7):502–14. https://doi.org/10.1038/nrm2927.

Hart, Roger J. 2016. "Physiological Aspects of Female Fertility: Role of the Environment, Modern Lifestyle, and Genetics." *Physiological Reviews* 96 (3):873–909. https://doi.org/10.1152/physrev.00023.2015.

Hatirnaz, Safak, Alev Ozer, Ebru Hatirnaz, Melahat Atasever, Serdar Başaranoglu, Mine Kanat-Pektas, and Murat Api. 2017. "Pre-Implantation Genetic Screening among Women Experiencing Recurrent Failure of in Vitro Fertilization." *International Journal of Gynaecology and Obstetrics: The Official Organ of the International Federation of Gynaecology and Obstetrics*, March. https://doi.org/10.1002/ijgo.12135.

Hayashi, Yohei, Kei Otsuka, Masayuki Ebina, Kaori Igarashi, Asuka Takehara, Mitsuyo Matsumoto, Akio Kanai, Kazuhiko Igarashi, Tomoyoshi Soga, and Yasuhisa Matsui. 2017. "Distinct Requirements for Energy Metabolism in Mouse Primordial Germ Cells and Their Reprogramming to Embryonic Germ Cells." *Proceedings of the National Academy of Sciences of the United States of America* 114 (31):8289–94. https://doi.org/10.1073/pnas.1620915114.

Hebisha, Sherif A., Banan A. Aboelazm, and H. N. Sallam. 2017. "GnRH Antagonist Cetrorelix Administration Before HCG for Protection of Ovarian Hyperstimulation Syndrome." *Journal of Obstetrics and Gynaecology of India* 67 (4):270–74. https://doi.org/10.1007/s13224-016-0952-5.

Heitmann, Ryan J., Micah J. Hill, Kevin S. Richter, Alan H. DeCherney, and Eric A. Widra. 2013. "The Simplified SART Embryo Scoring System Is Highly Correlated to Implantation and Live Birth in Single Blastocyst Transfers." *Journal of Assisted Reproduction and Genetics* 30 (4):563–67. https://doi.org/10.1007/s10815-013-9932-1.

Heng, Boon C, Catherine M Cowan, and Shubhayu Basu. 2009. "Comparison of Enzymatic and Non-Enzymatic Means of Dissociating Adherent Monolayers of Mesenchymal Stem Cells." *Biological Procedures Online* 11 (March):161–69. https://doi.org/10.1007/s12575-009-9001-4.

Hizanidis, Johanne, Nikos E. Kouvaris, Gorka Zamora-López, Zamora-López Gorka, Albert Díaz-Guilera, and Chris G. Antonopoulos. 2016. “Chimera-like States in Modular Neural Networks.” *Scientific Reports* 6 (January):19845. https://doi.org/10.1038/srep19845.

Hodes-Wertz, Brooke, Jamie Grifo, Shahin Ghadir, Brian Kaplan, Carl A. Laskin, Michael Glassner, and Santiago Munné. 2012. “Idiopathic Recurrent Miscarriage Is Caused Mostly by Aneuploid Embryos.” *Fertility and Sterility* 98 (3):675–80. https://doi.org/10.1016/j.fertnstert.2012.05.025.

Hodgen, G. D. 1982. “The Dominant Ovarian Follicle.” *Fertility and Sterility* 38 (3):281–300.

Huang, Jack Yu Jen, and Zev Rosenwaks. 2012. “In Vitro Fertilisation Treatment and Factors Affecting Success.” *Best Practice & Research. Clinical Obstetrics & Gynaecology* 26 (6):777–88. https://doi.org/10.1016/j.bpobgyn.2012.08.017.

Humaidan, P., S. M. Nelson, P. Devroey, C. C. Coddington, L. B. Schwartz, K. Gordon, J. L. Frattarelli, et al. 2016. “Ovarian Hyperstimulation Syndrome: Review and New Classification Criteria for Reporting in Clinical Trials.” *Human Reproduction (Oxford, England)*, June. https://doi.org/10.1093/humrep/dew149.

Hutson, J., H. Ikawa, and P. K. Donahoe. 1981. “The Ontogeny of Mullerian Inhibiting Substance in the Gonads of the Chicken.” *Journal of Pediatric Surgery* 16 (6):822–27.

Ivec, Martin, Borut Kovacic, and Veljko Vlaisavljevic. 2011. “Prediction of Human Blastocyst Development from Morulas with Delayed and/or Incomplete Compaction.” *Fertility and Sterility* 96 (6):1473–1478.e2. https://doi.org/10.1016/j.fertnstert.2011.09.015.

Johnson, D. S., G. Gemelos, J. Baner, A. Ryan, C. Cinnioglu, M. Banjevic, R. Ross, et al. 2010. “Preclinical Validation of a Microarray Method for Full Molecular Karyotyping of Blastomeres in a 24-h Protocol.” *Human Reproduction (Oxford, England)* 25 (4):1066–75. https://doi.org/10.1093/humrep/dep452.

Kawakami, Masayoshi, Masahide Inoue, and Joy M. Richman. 2006. “Cell Dissociation Experiments Reveal That Positional Information Operates in the Chicken Frontonasal Mass.” *Genesis (New York, N.Y.: 2000)* 44 (3):105–14. https://doi.org/10.1002/gene.20191.

Kayisli, Umit A., Belgin Selam, Ozlem Guzeloglu-Kayisli, Ramazan Demir, and Aydin Arici. 2003. “Human Chorionic Gonadotropin Contributes to Maternal Immunotolerance and Endometrial Apoptosis by Regulating Fas-Fas Ligand System.” *Journal of Immunology (Baltimore, Md.: 1950)* 171 (5):2305–13.

Kelley, Rebecca L., and David K. Gardner. 2016. “Combined Effects of Individual Culture and Atmospheric Oxygen on Preimplantation Mouse Embryos in Vitro.” *Reproductive Biomedicine Online* 33 (5):537–49. https://doi.org/10.1016/j.rbmo.2016.08.003.

———. 2017. “In Vitro Culture of Individual Mouse Preimplantation Embryos: The Role of Embryo Density, Microwells, Oxygen, Timing and Conditioned Media.” *Reproductive Biomedicine Online* 34 (5):441–54. https://doi.org/10.1016/j.rbmo.2017.02.001.

Keltz, Martin D., Mario Vega, Ido Sirota, Matthew Lederman, Erin L. Moshier, Eric Gonzales, and Daniel Stein. 2013. “Preimplantation Genetic Screening (PGS) with Comparative Genomic Hybridization (CGH) Following Day 3 Single Cell Blastomere Biopsy Markedly Improves IVF Outcomes While Lowering Multiple Pregnancies and Miscarriages.” *Journal of Assisted Reproduction and Genetics* 30 (10):1333–39. https://doi.org/10.1007/s10815-013-0070-6.

Ketha, Hemamalini, Sukhbir Kaur, Stefan K. Grebe, and Ravinder J. Singh. 2014. “Clinical Applications of LC-MS Sex Steroid Assays: Evolution of Methodologies in the 21st Century.” *Current Opinion in Endocrinology, Diabetes, and Obesity* 21 (3):217–26. https://doi.org/10.1097/MED.0000000000000068.

Khuri, A. 1999. “A Necessary Condition for a Quadratic Form to Have a Chi-Squared Distribution: An Accessible Proof.” *International Journal of Mathematical Education in Science and Technology* 30 (3):335–39. https://doi.org/10.1080/002073999287860.

Kidder, Benjamin L., Leann Oseth, Shanna Miller, Betsy Hirsch, Catherine Verfaillie, and Electra Coucouvanis. 2008. “Embryonic Stem Cells Contribute to Mouse Chimeras in the Absence of Detectable Cell Fusion.” *Cloning and Stem Cells* 10 (2):231–48. https://doi.org/10.1089/clo.2007.0039.

Kim, Hae-Young. 2014a. “Analysis of Variance (ANOVA) Comparing Means of More than Two Groups.” *Restorative Dentistry & Endodontics* 39 (1):74–77. https://doi.org/10.5395/rde.2014.39.1.74.

———. 2014b. “Statistical Notes for Clinical Researchers: Two-Way Analysis of Variance (ANOVA)-Exploring Possible Interaction between Factors.” *Restorative Dentistry & Endodontics* 39 (2):143–47. https://doi.org/10.5395/rde.2014.39.2.143.

Koal, Therese, Diane Schmiederer, Hai Pham-Tuan, Cornelia Röhring, and Manfred Rauh. 2012. “Standardized LC-MS/MS Based Steroid Hormone Profile-Analysis.” *The Journal of Steroid Biochemistry and Molecular Biology* 129 (3–5):129–38. https://doi.org/10.1016/j.jsbmb.2011.12.001.

Koskela, Sanna, and Juha Tapanainen. 2016. “[Anti-Müllerian hormone - a marker of ovarian function].” *Duodecim; Lääketieteellinen Aikakauskirja* 132 (3):226–32.

Kotdawala, Aditi, Deven Patel, Javier Herrero, Rajni Khajuria, Nalini Mahajan, and Manish Banker. 2016. “Aneuploidy Screening by Array Comparative Genomic Hybridization Improves Success Rates of in Vitro Fertilization: A Multicenter Indian Study.” *Journal of Human Reproductive Sciences* 9 (4):223–29. https://doi.org/10.4103/0974-1208.197630.

Kuhn, Max, and Kjell Johnson. 2013. *Applied Predictive Modeling*. New York, NY: Springer New York. https://doi.org/10.1007/978-1-4614-6849-3.

Lafond, J., M. Leclerc, and M. G. Brunette. 1991. “Characterization of Calcium Transport by Basal Plasma Membranes from Human Placental Syncytiotrophoblast.” *Journal of Cellular Physiology* 148 (1):17–23. https://doi.org/10.1002/jcp.1041480103.

Lai, Qiaohong, Hanwang Zhang, Guijing Zhu, Yufeng Li, Lei Jin, Long He, Zhijun Zhang, et al. 2013. “Comparison of the GnRH Agonist and Antagonist Protocol on the Same Patients in Assisted Reproduction during Controlled Ovarian Stimulation Cycles.” *International Journal of Clinical and Experimental Pathology* 6 (9):1903–10.

Lamazou, F., F. Fuchs, M. Grynberg, V. Gallot, E. Herzog, R. Fanchin, N. Frydman, and R. Frydman. 2012. “[Cancellation of IVF-ET cycles: poor prognosis, poor responder, or variability of the response to controlled ovarian hyperstimulation? An analysis of 142 cancellations].” *Journal De Gynécologie, Obstétrique Et Biologie De La Reproduction* 41 (1):41–47. https://doi.org/10.1016/j.jgyn.2011.06.001.

Lane, M., and David K. Gardner. 2000. “Regulation of Ionic Homeostasis by Mammalian Embryos.” *Seminars in Reproductive Medicine* 18 (2):195–204. https://doi.org/10.1055/s-2000-12558.

Lane, Michelle, Megan Mitchell, Kara S. Cashman, Deanne Feil, Sarah Wakefield, and Deirdre L. Zander-Fox. 2008. “To QC or Not to QC: The Key to a Consistent Laboratory?” *Reproduction, Fertility, and Development* 20 (1):23–32.

Lathi, Ruth B., Jamie A. M. Massie, Morgan Gilani, Amin A. Milki, Lynn M. Westphal, Valerie L. Baker, and Barry Behr. 2012. “Outcomes of Trophectoderm Biopsy on Cryopreserved Blastocysts: A Case Series.” *Reproductive Biomedicine Online* 25 (5):504–7. https://doi.org/10.1016/j.rbmo.2012.06.021.

Lee, Bang Hyun, Jae Hoon Kim, Tae Chul Park, and Hee Joong Lee. 2016. “Relationship Between Gonadotropin-Releasing Hormone/Gonadotropin-Releasing Hormone Receptor Signaling and Progesterone Receptors in Human Trophoblasts.” *The Journal of Reproductive Medicine* 61 (5–6):275–81.

Levi, A. J., M. F. Raynault, P. A. Bergh, M. R. Drews, B. T. Miller, and R. T. Scott. 2001. “Reproductive Outcome in Patients with Diminished Ovarian Reserve.” *Fertility and Sterility* 76 (4):666–69.

Li, Chao-Bo, Li-Li Hu, Zhen-Dong Wang, Shu-Qi Zhong, and Lei Lei. 2009. “[Regulation of compaction initiation in mouse embryo].” *Yi Chuan = Hereditas* 31 (12):1177–84.

Li, Gang, Haixia Jin, Zhimin Xin, Yingchun Su, Paul R. Brezina, Andrew T. Benner, William G. Kearns, and Yingpu Sun. 2014. “Increased IVF Pregnancy Rates after Microarray Preimplantation Genetic Diagnosis Due to Parental Translocations.” *Systems Biology in Reproductive Medicine* 60 (2):119–24. https://doi.org/10.3109/19396368.2013.875241.

Li, Yuanyuan, Ran Wang, Nan Qiao, Guangdun Peng, Ke Zhang, Ke Tang, Jing-Dong J. Han, and Naihe Jing. 2017. “Transcriptome Analysis Reveals Determinant Stages Controlling

Human Embryonic Stem Cell Commitment to Neuronal Cells." *The Journal of Biological Chemistry*, September. https://doi.org/10.1074/jbc.M117.796383.

Lim, C. Y., B. B. Knowles, D. Solter, and D. M. Messerschmidt. 2016. "Epigenetic Control of Early Mouse Development." *Current Topics in Developmental Biology* 120:311–60. https://doi.org/10.1016/bs.ctdb.2016.05.002.

Lindberg, Sara, Cynthia Anderson, Parvathy Pillai, Aman Tandias, Brian Arndt, and Lawrence Hanrahan. 2016. "Prevalence and Predictors of Unhealthy Weight Gain in Pregnancy." *WMJ: Official Publication of the State Medical Society of Wisconsin* 115 (5):233–37.

Liu, Hongbin, Qiwei Yang, Krishnan Radhakrishnan, Dedra E. Whitfield, Camille L. M. Everhart, Patricia Parsons-Wingerter, and Steven A. Fisher. 2009. "Role of VEGF and Tissue Hypoxia in Patterning of Neural and Vascular Cells Recruited to the Embryonic Heart." *Developmental Dynamics: An Official Publication of the American Association of Anatomists* 238 (11):2760–69. https://doi.org/10.1002/dvdy.22103.

Liu, Huimin. 2008. "Generalized Additive Model." *Department of Mathematics and Statistics, University of Minnesota Duluth, Duluth, MN* 55812. http://www.d.umn.edu/math/Technical%20Reports/Technical%20Reports%202007-/TR%202007-2008/TR_2008_8.pdf.

Liu, Jing, Francois Bernier, Julie Lauzon, R. Brian Lowry, and Judy Chernos. 2011. "Application of Microarray-Based Comparative Genomic Hybridization in Prenatal and Postnatal Settings: Three Case Reports." *Genetics Research International* 2011:976398. https://doi.org/10.4061/2011/976398.

Lledó, Belén, Ruth Morales, Jose Antonio Ortiz, Helena Blanca, Jorge Ten, Joaquín Llácer, and Rafael Bernabeu. 2017. "Implantation Potential of Mosaic Embryos." *Systems Biology in Reproductive Medicine* 63 (3):206–8. https://doi.org/10.1080/19396368.2017.1296045.

Los Santos, Maria José de, Pilar Gámiz, José María de Los Santos, Josep Lluís Romero, Nicolás Prados, Cristina Alonso, José Remohí, and Francisco Dominguez. 2015. "The Metabolomic Profile of Spent Culture Media from Day-3 Human Embryos Cultured under Low Oxygen Tension." *PloS One* 10 (11):e0142724. https://doi.org/10.1371/journal.pone.0142724.

Lowe, Jenna Louise, George Koustas, Cecilia Sjoblom, and Christopher Gerald Grupen. 2015. "A Comparison of Different Vitrification Devices and the Effect of Blastocoele Collapse on the Cryosurvival of in Vitro Produced Porcine Embryos." Edited by Louise Katherine Bartolac. *The Journal of Reproduction and Development* 61 (6):525–31. https://doi.org/10.1262/jrd.2015-065.

Łukaszuk, Krzysztof, Sebastian Pukszta, Dagan Wells, Celina Cybulska, Joanna Liss, Łukasz Płóciennik, Waldemar Kuczyński, and Judyta Zabielska. 2015. "Routine Use of Next-Generation Sequencing for Preimplantation Genetic Diagnosis of Blastomeres Obtained from Embryos on Day 3 in Fresh in Vitro Fertilization Cycles." *Fertility and Sterility* 103 (4):1031–36. https://doi.org/10.1016/j.fertnstert.2014.12.123.

Magli, M. Cristina, Alessandra Pomante, Giulia Cafueri, Marzia Valerio, Andor Crippa, Anna P. Ferraretti, and Luca Gianaroli. 2016. “Preimplantation Genetic Testing: Polar Bodies, Blastomeres, Trophectoderm Cells, or Blastocoelic Fluid?” *Fertility and Sterility* 105 (3):676–683.e5. https://doi.org/10.1016/j.fertnstert.2015.11.018.

Maître, Jean-Léon, and Carl-Philipp Heisenberg. 2013. “Three Functions of Cadherins in Cell Adhesion.” *Current Biology* 23 (14):R626–33. https://doi.org/10.1016/j.cub.2013.06.019.

Marshall, Charla, Kimberly Sturk-Andreaggi, Jennifer Daniels-Higginbotham, Robert Sean Oliver, Suzanne Barritt-Ross, and Timothy P. McMahon. 2017. “Performance Evaluation of a Mitogenome Capture and Illumina Sequencing Protocol Using Non-Probative, Case-Type Skeletal Samples: Implications for the Use of a Positive Control in a next-Generation Sequencing Procedure.” *Forensic Science International. Genetics* 31 (September):198–206. https://doi.org/10.1016/j.fsigen.2017.09.001.

Mathews, T. J., Brady E. Hamilton, National Center for Health Statistics (US), and others. 2009. “Delayed Childbearing: More Women Are Having Their First Child Later in Life.” http://blogs.sgmcb.com/glassceilinggrubbyfloor/research/NHCS%20Data%20Brief%201%20-%20Delayed%20Childbearing.pdf.

Meer, M. van der, P. G. Hompes, F. Scheele, E. Schoute, S. Veersema, and J. Schoemaker. 1994. “Follicle Stimulating Hormone (FSH) Dynamics of Low Dose Step-up Ovulation Induction with FSH in Patients with Polycystic Ovary Syndrome.” *Human Reproduction (Oxford, England)* 9 (9):1612–17.

Meldrum, David R., Robert F. Casper, Antonio Diez-Juan, Carlos Simon, Alice D. Domar, and Rene Frydman. 2016. “Aging and the Environment Affect Gamete and Embryo Potential: Can We Intervene?” *Fertility and Sterility* 105 (3):548–59. https://doi.org/10.1016/j.fertnstert.2016.01.013.

Meller, R. 2015. “Addressing Benefits, Risks and Consent in Next Generation Sequencing Studies.” *Journal of Clinical Research & Bioethics* 6 (6). https://doi.org/10.4172/2155-9627.1000249.

Menze, Bjoern H, B Michael Kelm, Ralf Masuch, Uwe Himmelreich, Peter Bachert, Wolfgang Petrich, and Fred A Hamprecht. 2009. “A Comparison of Random Forest and Its Gini Importance with Standard Chemometric Methods for the Feature Selection and Classification of Spectral Data.” *BMC Bioinformatics* 10 (July):213. https://doi.org/10.1186/1471-2105-10-213.

Mihajlović, Aleksandar I., Vasanth Thamodaran, and Alexander W. Bruce. 2015. “The First Two Cell-Fate Decisions of Preimplantation Mouse Embryo Development Are Not Functionally Independent.” *Scientific Reports* 5:15034. https://doi.org/10.1038/srep15034.

Minooee, S., F. Ramezani Tehrani, M. Rahmati, M. Ali Mansournia, and F. Azizi. 2017. “Prediction of Age at Menopause in Women with Polycystic Ovary Syndrome.”

*Climacteric: The Journal of the International Menopause Society*, November, 1–6. https://doi.org/10.1080/13697137.2017.1392501.

Mioni, Roberto, and Giuseppe Mioni. 2015. "A Mathematical Model of PH, Based on the Total Stoichiometric Concentration of Acids, Bases and Ampholytes Dissolved in Water." *Scandinavian Journal of Clinical and Laboratory Investigation* 75 (6):452–69. https://doi.org/10.3109/00365513.2015.1042402.

Morbeck, Dean E., Nikola A. Baumann, and Devin Oglesbee. 2017. "Composition of Single-Step Media Used for Human Embryo Culture." *Fertility and Sterility* 107 (4):1055–1060.e1. https://doi.org/10.1016/j.fertnstert.2017.01.007.

Morgante, G., M. G. Massaro, A. Di Sabatino, V. Cappelli, and V. De Leo. 2017. "Therapeutic Approach for Metabolic Disorders and Infertility in Women with PCOS." *Gynecological Endocrinology: The Official Journal of the International Society of Gynecological Endocrinology*, August, 1–6. https://doi.org/10.1080/09513590.2017.1370644.

Muasher, S. J., S. Oehninger, S. Simonetti, J. Matta, L. M. Ellis, H. C. Liu, G. S. Jones, and Z. Rosenwaks. 1988. "The Value of Basal and/or Stimulated Serum Gonadotropin Levels in Prediction of Stimulation Response and in Vitro Fertilization Outcome." *Fertility and Sterility* 50 (2):298–307.

Mugnier, Sylvie, Morgane Kervella, Cécile Douet, Sylvie Canepa, Géraldine Pascal, Stefan Deleuze, Guy Duchamp, Philippe Monget, and Ghylène Goudet. 2009. "The Secretions of Oviduct Epithelial Cells Increase the Equine in Vitro Fertilization Rate: Are Osteopontin, Atrial Natriuretic Peptide A and Oviductin Involved?" *Reproductive Biology and Endocrinology : RB&E* 7 (November):129. https://doi.org/10.1186/1477-7827-7-129.

Mui, Keeley L., Christopher S. Chen, and Richard K. Assoian. 2016. "The Mechanical Regulation of Integrin-Cadherin Crosstalk Organizes Cells, Signaling and Forces." *Journal of Cell Science* 129 (6):1093–1100. https://doi.org/10.1242/jcs.183699.

Mumusoglu, Sezcan, Irem Yarali, Gurkan Bozdag, Pınar Ozdemir, Mehtap Polat, Lale Karakoc Sokmensuer, and Hakan Yarali. 2017. "Time-Lapse Morphokinetic Assessment Has Low to Moderate Ability to Predict Euploidy When Patient- and Ovarian Stimulation-Related Factors Are Taken into Account with the Use of Clustered Data Analysis." *Fertility and Sterility* 107 (2):413–421.e4. https://doi.org/10.1016/j.fertnstert.2016.11.005.

Munné, S., A. Lee, Z. Rosenwaks, J. Grifo, and J. Cohen. 1993. "Diagnosis of Major Chromosome Aneuploidies in Human Preimplantation Embryos." *Human Reproduction (Oxford, England)* 8 (12):2185–91.

Munné, Santiago, and Dagan Wells. 2017. "Detection of Mosaicism at Blastocyst Stage with the Use of High-Resolution next-Generation Sequencing." *Fertility and Sterility* 107 (5):1085–91. https://doi.org/10.1016/j.fertnstert.2017.03.024.

Muzzey, Dale, Eric A. Evans, and Caroline Lieber. 2015. “Understanding the Basics of NGS: From Mechanism to Variant Calling.” *Current Genetic Medicine Reports* 3 (4):158–65. https://doi.org/10.1007/s40142-015-0076-8.

Nagy, Zsolt Peter, Alex C. Varghese, and Ashok Agarwal. 2012. *Practical Manual of In Vitro Fertilization: Advanced Methods and Novel Devices*. Springer Science & Business Media.

Namwanje, Maria, and Chester W. Brown. 2016. “Activins and Inhibins: Roles in Development, Physiology, and Disease.” *Cold Spring Harbor Perspectives in Biology* 8 (7). https://doi.org/10.1101/cshperspect.a021881.

Nastri, Carolina O., Beatrice N. Nóbrega, Danielle M. Teixeira, Jowanka Amorim, Lívia M. M. Diniz, Marina W. P. Barbosa, Vanessa S. I. Giorgi, Vicky N. Pileggi, and Wellington P. Martins. 2016. “Low versus Atmospheric Oxygen Tension for Embryo Culture in Assisted Reproduction: A Systematic Review and Meta-Analysis.” *Fertility and Sterility*, March. https://doi.org/10.1016/j.fertnstert.2016.02.037.

Natale, Bryony V., Christina Schweitzer, Martha Hughes, Maria A. Globisch, Ramie Kotadia, Emilie Tremblay, Priscilla Vu, James C. Cross, and David R. C. Natale. 2017. “Sca-1 Identifies a Trophoblast Population with Multipotent Potential in the Mid-Gestation Mouse Placenta.” *Scientific Reports* 7 (1):5575. https://doi.org/10.1038/s41598-017-06008-2.

“National ART Summary Report for 2013.” n.d. Accessed February 17, 2016. http://www.cdc.gov/art/pdf/2013-report/art_2013_national_summary_report.pdf#page=11.

“National Summary Report | 2013 ART Report | Division of Reproductive Health | CDC.” 2015. October 2015. http://www.cdc.gov/art/reports/2013/national-summary.html.

Niakan, Kathy K., Jinnuo Han, Roger A. Pedersen, Carlos Simon, and Renee A. Reijo Pera. 2012. “Human Pre-Implantation Embryo Development.” *Development (Cambridge, England)* 139 (5):829–41. https://doi.org/10.1242/dev.060426.

Nikas, G., A. Ao, R. M. Winston, and A. H. Handyside. 1996. “Compaction and Surface Polarity in the Human Embryo in Vitro.” *Biology of Reproduction* 55 (1):32–37.

Nyboe Andersen, Anders, Scott M. Nelson, Bart C. J. M. Fauser, Juan Antonio García-Velasco, Bjarke M. Klein, and Joan-Carles Arce. 2017. “Individualized versus Conventional Ovarian Stimulation for in Vitro Fertilization: A Multicenter, Randomized, Controlled, Assessor-Blinded, Phase 3 Noninferiority Trial.” *Fertility and Sterility* 107 (2):387–396.e4. https://doi.org/10.1016/j.fertnstert.2016.10.033.

Oehninger, S. 2011. “Poor Responders in in Vitro Fertilization (IVF) Therapy: The Challenge Continues.” *Facts, Views & Vision in ObGyn* 3 (2):101–8.

Ohnuma, Kiyoshi, Ayaka Fujiki, Kana Yanagihara, Saoko Tachikawa, Yohei Hayashi, Yuzuru Ito, Yasuko Onuma, et al. 2014. “Enzyme-Free Passage of Human Pluripotent Stem Cells by Controlling Divalent Cations.” *Scientific Reports* 4 (April). https://doi.org/10.1038/srep04646.

Osadchuk, L. V., A. V. Popova, A. A. Erkovich, N. A. Voroshilova, and A. V. Osadchuk. 2017. “[Effects of smoking and alcohol consumptionon reproductive and metabolic indicators in young men in western siberia].” *Urologiia (Moscow, Russia: 1999)*, no. 4 (September):62–67.

Oudshoorn, Simone C., Van Tilborg, Theodora C, Marinus J. C. Eijkemans, G. Jur E. Oosterhuis, Jaap Friederich, Van Hooff, et al. 2017. “Individualized versus Standard FSH Dosing in Women Starting IVF/ICSI: An RCT. Part 2: The Predicted Hyper Responder.” *Human Reproduction*, February, 1–9. https://doi.org/10.1093/humrep/dex319.

Palacios, Felipe, Jogender S. Tushir, Yasuyuki Fujita, and Crislyn D’Souza-Schorey. 2005. “Lysosomal Targeting of E-Cadherin: A Unique Mechanism for the down-Regulation of Cell-Cell Adhesion during Epithelial to Mesenchymal Transitions.” *Molecular and Cellular Biology* 25 (1):389–402. https://doi.org/10.1128/MCB.25.1.389-402.2005.

Pandis, Nikolaos. 2016. “The Chi-Square Test.” *American Journal of Orthodontics and Dentofacial Orthopedics* 150 (5):898–99. https://doi.org/10.1016/j.ajodo.2016.08.009.

Peng, Zhao-Feng, Sen-Lin Shi, Hai-Xia Jin, Gui-Dong Yao, En-Yin Wang, Hong-Yi Yang, Wen-Yan Song, and Ying-Pu Sun. 2015. “Impact of Oxygen Concentrations on Fertilization, Cleavage, Implantation, and Pregnancy Rates of in Vitro Generated Human Embryos.” *International Journal of Clinical and Experimental Medicine* 8 (4):6179–85.

Perkel, Kayla J., and Pavneesh Madan. 2017. “Spent Culture Medium Analysis from Individually Cultured Bovine Embryos Demonstrates Metabolomic Differences.” *Zygote (Cambridge, England)*, October, 1–13. https://doi.org/10.1017/S0967199417000417.

Piccolomini, Mariana M., Mariana Nicolielo, Tatiana C. S. Bonetti, Eduardo L. A. Motta, Paulo C. Serafini, and Jose Roberto Alegretti. 2016. “Does Slow Embryo Development Predict a High Aneuploidy Rate on Trophectoderm Biopsy?” *Reproductive Biomedicine Online*, June. https://doi.org/10.1016/j.rbmo.2016.06.005.

Plouhinec, Jean-Louis, and E. M. De Robertis. 2007. “Systems Biology of Embryonic Morphogens.” *Molecular BioSystems* 3 (7):454–57. https://doi.org/10.1039/b701571b.

Pollard, A. S., B. G. Charlton, J. R. Hutchinson, T. Gustafsson, I. M. McGonnell, J. A. Timmons, and A. A. Pitsillides. 2017. “Limb Proportions Show Developmental Plasticity in Response to Embryo Movement.” *Scientific Reports* 7 (February):srep41926. https://doi.org/10.1038/srep41926.

Pouwer, Annefloor W., Cindy Farquhar, and Jan A. M. Kremer. 2015. “Long-Acting FSH versus Daily FSH for Women Undergoing Assisted Reproduction.” *The Cochrane Database of*

*Systematic Reviews*, no. 7:CD009577. https://doi.org/10.1002/14651858.CD009577.pub3.

Powers, David Martin. 2011. "Evaluation: From Precision, Recall and F-Measure to ROC, Informedness, Markedness and Correlation."

Price, Trevor D, Anna Qvarnström, and Darren E Irwin. 2003. "The Role of Phenotypic Plasticity in Driving Genetic Evolution." *Proceedings of the Royal Society B: Biological Sciences* 270 (1523):1433–40. https://doi.org/10.1098/rspb.2003.2372.

Priya, Rashmi, and Alpha S. Yap. 2015. "Making a Choice: How Cadherin Switching Controls Cell Migration." *Developmental Cell* 34 (4):383–84. https://doi.org/10.1016/j.devcel.2015.08.002.

Racowsky, Catherine, Michael Vernon, Jacob Mayer, G. David Ball, Barry Behr, Kimball O. Pomeroy, David Wininger, William Gibbons, Joseph Conaghan, and Judy E. Stern. 2010. "Standardization of Grading Embryo Morphology." *Journal of Assisted Reproduction and Genetics* 27 (8):437–39. https://doi.org/10.1007/s10815-010-9443-2.

Ralston, Amy, and Janet Rossant. 2010. "The Genetics of Induced Pluripotency." *Reproduction (Cambridge, England)* 139 (1):35–44. https://doi.org/10.1530/REP-09-0024.

Reijo Pera, R. A., and L. Prezzoto. 2016. "Species-Specific Variation Among Mammals." *Current Topics in Developmental Biology* 120:401–20. https://doi.org/10.1016/bs.ctdb.2016.05.003.

Ren, Hui, Nigel P. Birch, and Vinod Suresh. 2016. "An Optimised Human Cell Culture Model for Alveolar Epithelial Transport." *PloS One* 11 (10):e0165225. https://doi.org/10.1371/journal.pone.0165225.

Reproductive Endocrinology and Infertility Committee, Family Physicians Advisory Committee, Maternal-Fetal Medicine Committee, Executive and Council of the Society of Obstetricians, Kimberly Liu, and Allison Case. 2011. "Advanced Reproductive Age and Fertility." *Journal of Obstetrics and Gynaecology Canada: JOGC = Journal d'obstétrique et Gynécologie Du Canada: JOGC* 33 (11):1165–75.

Rinaldi, Leonardo, and Helmy Selman. 2016. "Profile of Follitropin Alpha/Lutropin Alpha Combination for the Stimulation of Follicular Development in Women with Severe Luteinizing Hormone and Follicle-Stimulating Hormone Deficiency." *International Journal of Women's Health* 8:169–79. https://doi.org/10.2147/IJWH.S88904.

Robin, Xavier, Natacha Turck, Alexandre Hainard, Natalia Tiberti, Frédérique Lisacek, Jean-Charles Sanchez, and Markus Müller. 2011. "PROC: An Open-Source Package for R and S+ to Analyze and Compare ROC Curves." *BMC Bioinformatics* 12 (1):77.

Rooij, Ilse A. J. van, Frank J. M. Broekmans, Gabrielle J. Scheffer, Caspar W. N. Looman, J. Dik F. Habbema, Frank H. de Jong, Bart J. C. M. Fauser, Axel P. N. Themmen, and Egbert R. te Velde. 2005. "Serum Antimullerian Hormone Levels Best Reflect the

Reproductive Decline with Age in Normal Women with Proven Fertility: A Longitudinal Study." *Fertility and Sterility* 83 (4):979–87. https://doi.org/10.1016/j.fertnstert.2004.11.029.

Ruane, Peter T., Stéphane C. Berneau, Rebekka Koeck, Jessica Watts, Susan J. Kimber, Daniel R. Brison, Melissa Westwood, and John D. Aplin. 2017. "Apposition to Endometrial Epithelial Cells Activates Mouse Blastocysts for Implantation." *MHR: Basic Science of Reproductive Medicine* 23 (9):617–27. https://doi.org/10.1093/molehr/gax043.

Rubio, C., C. Simón, F. Vidal, L. Rodrigo, T. Pehlivan, J. Remohí, and A. Pellicer. 2003. "Chromosomal Abnormalities and Embryo Development in Recurrent Miscarriage Couples." *Human Reproduction (Oxford, England)* 18 (1):182–88.

Rubio, Carmen, Lorena Rodrigo, Pere Mir, Emilia Mateu, Vanessa Peinado, Miguel Milán, Nasser Al-Asmar, Inmaculada Campos-Galindo, Sandra Garcia, and Carlos Simón. 2013. "Use of Array Comparative Genomic Hybridization (Array-CGH) for Embryo Assessment: Clinical Results." *Fertility and Sterility* 99 (4):1044–48. https://doi.org/10.1016/j.fertnstert.2013.01.094.

Ruvinsky, Igor, Maximiliano Katz, Avigail Dreazen, Yuval Gielchinsky, Ann Saada, Nanette Freedman, Eyal Mishani, Gabriel Zimmerman, Judith Kasir, and Oded Meyuhas. 2009. "Mice Deficient in Ribosomal Protein S6 Phosphorylation Suffer from Muscle Weakness That Reflects a Growth Defect and Energy Deficit." *PloS One* 4 (5):e5618. https://doi.org/10.1371/journal.pone.0005618.

Ryley, D. A., D. Ezcurra, T. Von Wald, A. Zimon, and M. M. Alper. 2005. "IVF Success Rates in Patients With an Elevated Baseline FSH (> 10 MIU/Ml): Antagonist vs. Microdose Flare." *Fertility and Sterility* 84 (September):S43–44. https://doi.org/10.1016/j.fertnstert.2005.07.103.

Safdarian, Leili, Zahra Khayatzadeh, Ebrahim Djavadi, Atossa Mahdavi, Marzieh Aghahosseini, Ashraf Aleyasin, Parvin Fallahi, Sima Khayatzadeh, Arash Ahmadzadeh, and Mohhamad Bagher Larijani. 2012. "Prediction of Assisted Reproductive Technique Outcome in Elevated Early Follicular Phase Follicle Stimulating Hormone with Mullerian Inhibiting Substance Level." *Iranian Journal of Reproductive Medicine* 10 (3):201–8.

Sagrillo-Fagundes, Lucas, Hélène Clabault, Laetitia Laurent, Andrée-Anne Hudon-Thibeault, Eugênia Maria Assunção Salustiano, Marlène Fortier, Josianne Bienvenue-Pariseault, Philippe Wong Yen, J. Thomas Sanderson, and Cathy Vaillancourt. 2016. "Human Primary Trophoblast Cell Culture Model to Study the Protective Effects of Melatonin Against Hypoxia/Reoxygenation-Induced Disruption." *Journal of Visualized Experiments: JoVE*, no. 113 (July). https://doi.org/10.3791/54228.

Saito, Shigeo, Ying-Chu Lin, Ming-Ho Tsai, Chang-Shen Lin, Yoshinobu Murayama, Ryuji Sato, and Kazunari K. Yokoyama. 2015. "Emerging Roles of Hypoxia-Inducible Factors and Reactive Oxygen Species in Cancer and Pluripotent Stem Cells." *The Kaohsiung Journal of Medical Sciences* 31 (6):279–86. https://doi.org/10.1016/j.kjms.2015.03.002.

Saldarriaga, Wilmar, Flora Tassone, Laura Yuriko González-Teshima, Jose Vicente Forero-Forero, Sebastián Ayala-Zapata, and Randi Hagerman. 2014. “Fragile X Syndrome.” *Colombia Médica : CM* 45 (4):190–98.

Santoro, Michael R., Steven M. Bray, and Stephen T. Warren. 2012. “Molecular Mechanisms of Fragile X Syndrome: A Twenty-Year Perspective.” *Annual Review of Pathology* 7:219–45. https://doi.org/10.1146/annurev-pathol-011811-132457.

Sauer, Mark V. 2015. “Reproduction at an Advanced Maternal Age and Maternal Health.” *Fertility and Sterility* 103 (5):1136–43. https://doi.org/10.1016/j.fertnstert.2015.03.004.

Sauerbrun-Cutler, May-Tal, Mario Vega, Andrzej Breborowicz, Eric Gonzales, Daniel Stein, Mathew Lederman, and Martin Keltz. 2015. “Oocyte Zona Pellucida Dysmorphology Is Associated with Diminished In-Vitro Fertilization Success.” *Journal of Ovarian Research* 8 (February):5. https://doi.org/10.1186/s13048-014-0111-5.

Scheele, F., and J. Schoemaker. 1996. “The Role of Follicle-Stimulating Hormone in the Selection of Follicles in Human Ovaries: A Survey of the Literature and a Proposed Model.” *Gynecological Endocrinology: The Official Journal of the International Society of Gynecological Endocrinology* 10 (1):55–66.

Schroeder, Christopher, Marc Sturm, Andreas Dufke, Ulrike Mau-Holzmann, Thomas Eggermann, Sven Poths, Olaf Riess, and Michael Bonin. 2013. “UPDtool: A Tool for Detection of Iso- and Heterodisomy in Parent-Child Trios Using SNP Microarrays.” *Bioinformatics (Oxford, England)* 29 (12):1562–64. https://doi.org/10.1093/bioinformatics/btt174.

Sermon, Karen, Antonio Capalbo, Jacques Cohen, Edith Coonen, Martine De Rycke, Anick De Vos, Joy Delhanty, et al. 2016. “The Why, the How and the When of PGS 2.0: Current Practices and Expert Opinions of Fertility Specialists, Molecular Biologists, and Embryologists.” *Molecular Human Reproduction*, June. https://doi.org/10.1093/molehr/gaw034.

Shahbazi, Marta N., Agnieszka Jedrusik, Sanna Vuoristo, Gaelle Recher, Anna Hupalowska, Virginia Bolton, Norah M. E. Fogarty, et al. 2016. “Self-Organization of the Human Embryo in the Absence of Maternal Tissues.” *Nature Cell Biology* 18 (6):700–708. https://doi.org/10.1038/ncb3347.

Shahine, Lora K., Lorna Marshall, Julie D. Lamb, and Lee R. Hickok. 2016. “Higher Rates of Aneuploidy in Blastocysts and Higher Risk of No Embryo Transfer in Recurrent Pregnancy Loss Patients with Diminished Ovarian Reserve Undergoing in Vitro Fertilization.” *Fertility and Sterility*, June. https://doi.org/10.1016/j.fertnstert.2016.06.016.

Shi, Min, David M Umbach, and Clarice R Weinberg. 2013. “Case–sibling Studies That Acknowledge Unstudied Parents and Permit the Inclusion of Unmatched Individuals.” *International Journal of Epidemiology* 42 (1):298–307. https://doi.org/10.1093/ije/dys212.

Sifer, Christophe, Deborah Handelsman, Emilie Grange, Raphael Porcher, Christophe Poncelet, Brigitte Martin-Pont, Brigitte Benzacken, and Jean-Philippe Wolf. 2009. “An Auto-Controlled Prospective Comparison of Two Embryos Culture Media (G III Series versus ISM) for IVF and ICSI Treatments.” *Journal of Assisted Reproduction and Genetics* 26 (11–12):575–81. https://doi.org/10.1007/s10815-009-9357-z.

Simopoulou, Maria, Elena Nikolopoulou, Andreas Dimakakos, Konstantinos Charalabopoulos, and Michael Koutsilieris. 2014. “Cell Adhesion Molecules and in Vitro Fertilization.” *In Vivo (Athens, Greece)* 28 (5):683–90.

Singh, Nisha, Pawan Kumar Jayaswal, Kabita Panda, Paritra Mandal, Vinod Kumar, Balwant Singh, Shefali Mishra, et al. 2015. “Single-Copy Gene Based 50 K SNP Chip for Genetic Studies and Molecular Breeding in Rice.” *Scientific Reports* 5 (June):11600. https://doi.org/10.1038/srep11600.

Siristatidis, Charalampos S., Ahmed Gibreel, George Basios, Abha Maheshwari, and Siladitya Bhattacharya. 2015. “Gonadotrophin-Releasing Hormone Agonist Protocols for Pituitary Suppression in Assisted Reproduction.” *The Cochrane Database of Systematic Reviews*, no. 11 (November):CD006919. https://doi.org/10.1002/14651858.CD006919.pub4.

Smitz, Johan, Claudio Wolfenson, Scott Chappel, and Jane Ruman. 2016. “Follicle-Stimulating Hormone: A Review of Form and Function in the Treatment of Infertility.” *Reproductive Sciences (Thousand Oaks, Calif.)* 23 (6):706–16. https://doi.org/10.1177/1933719115607992.

Solter, Davor. 2010. “Viable Rat-Mouse Chimeras: Where Do We Go from Here?” *Cell* 142 (5):676–78. https://doi.org/10.1016/j.cell.2010.08.021.

Soundararajan, Rama, Anurag N. Paranjape, Valentin Barsan, Jeffrey T. Chang, and Sendurai A. Mani. 2015. “A Novel Embryonic Plasticity Gene Signature That Predicts Metastatic Competence and Clinical Outcome.” *Scientific Reports* 5 (June):srep11766. https://doi.org/10.1038/srep11766.

Sousa, Mário, Mariana Cunha, José Teixeira da Silva, Cristiano Oliveira, Joaquina Silva, Paulo Viana, and Alberto Barros. 2015. “Ovarian Hyperstimulation Syndrome: A Clinical Report on 4894 Consecutive ART Treatment Cycles.” *Reproductive Biology and Endocrinology: RB&E* 13:66. https://doi.org/10.1186/s12958-015-0067-3.

Speleman, F., N. Van Roy, J. Wiegant, M. R. Verschraegen-Spae, Y. Benoit, P. Govaert, L. Goossens, and J. G. Leroy. 1992. “Detection of Subtle Reciprocal Translocations by Fluorescence in Situ Hybridization.” *Clinical Genetics* 41 (4):169–74.

Staessen, C., W. Verpoest, P. Donoso, P. Haentjens, J. Van der Elst, I. Liebaers, and P. Devroey. 2008. “Preimplantation Genetic Screening Does Not Improve Delivery Rate in Women under the Age of 36 Following Single-Embryo Transfer.” *Human Reproduction (Oxford, England)* 23 (12):2818–25. https://doi.org/10.1093/humrep/den367.

Starkweather, Jon, and Amanda Kay Moske. 2011. “Multinomial Logistic Regression.” *Consulted Page at September 10th: Http://Www. Unt. Edu/Rss/Class/Jon/Benchmarks/MLR_JDS_Aug2011. Pdf* 29:2825–2830.

Steel, T., and J. Conaghan. 2008. “PH Equilibration Dynamics of Culture Medium Under Oil.” *Fertility and Sterility* 89 (4):S27. https://doi.org/10.1016/j.fertnstert.2008.02.084.

Steptoe, Andrew. 2015. “Biology: Changing the World - a Tribute to Patrick Steptoe, Robert Edwards and Jean Purdy.” *Human Fertility (Cambridge, England)* 18 (4):232–33. https://doi.org/10.3109/14647273.2015.1077657.

Stoop, Dominic, Ana Cobo, and Sherman Silber. 2014. “Fertility Preservation for Age-Related Fertility Decline.” *Lancet (London, England)* 384 (9950):1311–19. https://doi.org/10.1016/S0140-6736(14)61261-7.

Sunderam, Saswati, Dmitry M. Kissin, Sara B. Crawford, Suzanne G. Folger, Denise J. Jamieson, Lee Warner, and Wanda D. Barfield. 2017. “Assisted Reproductive Technology Surveillance - United States, 2014.” *Morbidity and Mortality Weekly Report. Surveillance Summaries (Washington, D.C.: 2002)* 66 (6):1–24. https://doi.org/10.15585/mmwr.ss6606a1.

Surrey, Eric S. 2007. “Management of the Poor Responder: The Role of GnRH Agonists and Antagonists.” *Journal of Assisted Reproduction and Genetics* 24 (12):613–19. https://doi.org/10.1007/s10815-007-9180-3.

Swain, J. E. 2010. “Optimizing the Culture Environment in the IVF Laboratory: Impact of PH and Buffer Capacity on Gamete and Embryo Quality.” *Reproductive BioMedicine Online* 21 (1):6–16. https://doi.org/10.1016/j.rbmo.2010.03.012.

———. 2012. “Is There an Optimal PH for Culture Media Used in Clinical IVF?” *Human Reproduction Update* 18 (3):333–39. https://doi.org/10.1093/humupd/dmr053.

Swain, J. E., L. Cabrera, X. Xu, and G. D. Smith. 2012. “Microdrop Preparation Factors Influence Culture-Media Osmolality, Which Can Impair Mouse Embryo Preimplantation Development.” *Reproductive Biomedicine Online* 24 (2):142–47. https://doi.org/10.1016/j.rbmo.2011.10.008.

Swain, J.E. 2015. “Optimal Human Embryo Culture.” *Seminars in Reproductive Medicine* 33 (2):103–17. https://doi.org/10.1055/s-0035-1546423.

Swain, J.E., Doug Carrell, Ana Cobo, Marcos Meseguer, Carmen Rubio, and Gary D. Smith. 2016. “Optimizing the Culture Environment and Embryo Manipulation to Help Maintain Embryo Developmental Potential.” *Fertility and Sterility* 105 (3):571–87. https://doi.org/10.1016/j.fertnstert.2016.01.035.

Tal, Reshef, Oded Tal, Benjamin J. Seifer, and David B. Seifer. 2015. “Antimüllerian Hormone as Predictor of Implantation and Clinical Pregnancy after Assisted Conception: A

Systematic Review and Meta-Analysis." *Fertility and Sterility* 103 (1):119–130.e3. https://doi.org/10.1016/j.fertnstert.2014.09.041.

Teh, Wan-Tinn, John McBain, and Peter Rogers. 2016. "What Is the Contribution of Embryo-Endometrial Asynchrony to Implantation Failure?" *Journal of Assisted Reproduction and Genetics*, August. https://doi.org/10.1007/s10815-016-0773-6.

Tejera, Alberto, Javier Herrero, Thamara Viloria, Josep Lluis Romero, Pilar Gamiz, and Marcos Meseguer. 2012. "Time-Dependent O2 Consumption Patterns Determined Optimal Time Ranges for Selecting Viable Human Embryos." *Fertility and Sterility* 98 (4):849-857.e1-3. https://doi.org/10.1016/j.fertnstert.2012.06.040.

Thompson, J. G., R. J. Partridge, F. D. Houghton, C. I. Cox, and H. J. Leese. 1996. "Oxygen Uptake and Carbohydrate Metabolism by in Vitro Derived Bovine Embryos." *Journal of Reproduction and Fertility* 106 (2):299–306.

Tilborg, Theodora C. van, Frank J. M. Broekmans, Madeleine Dólleman, Marinus J. C. Eijkemans, Ben Willem Mol, Joop S. E. Laven, and Helen L. Torrance. 2016. "Individualized Follicle-Stimulating Hormone Dosing and in Vitro Fertilization Outcome in Agonist Downregulated Cycles: A Systematic Review." *Acta Obstetricia Et Gynecologica Scandinavica* 95 (12):1333–44. https://doi.org/10.1111/aogs.13032.

Toh, Yi-Chin, Jiangwa Xing, and Hanry Yu. 2015. "Modulation of Integrin and E-Cadherin-Mediated Adhesions to Spatially Control Heterogeneity in Human Pluripotent Stem Cell Differentiation." *Biomaterials* 50 (May):87–97. https://doi.org/10.1016/j.biomaterials.2015.01.019.

Tomazevic, T., S. Korosec, I. Virant Klun, S. Drobnic, and I. Verdenik. 2007. "Age, Oestradiol and Blastocysts Can Predict Success in Natural Cycle IVF-Embryo Transfer." *Reproductive Biomedicine Online* 15 (2):220–26.

Treff, Nathan R., Jing Su, Xin Tao, Brynn Levy, and Richard T. Scott. 2010. "Accurate Single Cell 24 Chromosome Aneuploidy Screening Using Whole Genome Amplification and Single Nucleotide Polymorphism Microarrays." *Fertility and Sterility* 94 (6):2017–21. https://doi.org/10.1016/j.fertnstert.2010.01.052.

Ugoni, Antony, and Bruce F. Walker. 1995. "THE CHI SQUARE TEST." *COMSIG Review* 4 (3):61–64.

Valli, Roberto, Francesca De Bernardi, Annalisa Frattini, Luca Volpi, Maurizio Bignami, Fabio Facchetti, Francesco Pasquali, Paolo Castelnuovo, and Emanuela Maserati. 2015. "Comparative Genomic Hybridization on Microarray (a-CGH) in Olfactory Neuroblastoma: Analysis of Ten Cases and Review of the Literature." *Genes, Chromosomes & Cancer* 54 (12):771–75. https://doi.org/10.1002/gcc.22288.

Van Blerkom, Jonathan, Willem Ombelet, Elke Klerkx, Mia Janssen, Nathalie Dhont, Geeta Nargund, and Rudi Campo. 2014. "First Births with a Simplified Culture System for

Clinical IVF and Embryo Transfer." *Reproductive Biomedicine Online* 28 (3):310–20. https://doi.org/10.1016/j.rbmo.2013.11.012.

Vega, Mario G., Norbert Gleicher, Sarah K. Darmon, Andrea Weghofer, Yan-Guang Wu, Qi Wang, Lin Zhang, David F. Albertini, David H. Barad, and Vitaly A. Kushnir. 2016. "IVF Outcomes in Average- and Poor-Prognosis Infertile Women According to the Number of Embryos Transferred." *Reproductive Biomedicine Online*, June. https://doi.org/10.1016/j.rbmo.2016.06.009.

Vegetti, Walter, and Federica Alagna. 2006. "FSH and Folliculogenesis: From Physiology to Ovarian Stimulation." *Reproductive Biomedicine Online* 12 (6):684–94.

Veleva, Zdravka, Mauri Orava, Sinikka Nuojua-Huttunen, Juha S. Tapanainen, and Hannu Martikainen. 2013. "Factors Affecting the Outcome of Frozen-Thawed Embryo Transfer." *Human Reproduction (Oxford, England)* 28 (9):2425–31. https://doi.org/10.1093/humrep/det251.

Vet, Annemarie de, Joop S. E. Laven, Frank H. de Jong, Axel P. N. Themmen, and Bart C. J. M. Fauser. 2002. "Antimüllerian Hormone Serum Levels: A Putative Marker for Ovarian Aging." *Fertility and Sterility* 77 (2):357–62.

Visser, Jenny A., and Axel P. N. Themmen. 2014. "Role of Anti-Müllerian Hormone and Bone Morphogenetic Proteins in the Regulation of FSH Sensitivity." *Molecular and Cellular Endocrinology* 382 (1):460–65. https://doi.org/10.1016/j.mce.2013.08.012.

Visser, Marloes, Dennis Dooijes, Jasper J. van der Smagt, Jeroen F. van der Heijden, Pieter A. Doevendans, Peter Loh, Folkert W. Asselbergs, and Rutger J. Hassink. 2017. "Next-Generation Sequencing of a Large Gene Panel in Patients Initially Diagnosed with Idiopathic Ventricular Fibrillation." *Heart Rhythm* 14 (7):1035–40. https://doi.org/10.1016/j.hrthm.2017.01.010.

Volinsky, C. T., and A. E. Raftery. 2000. "Bayesian Information Criterion for Censored Survival Models." *Biometrics* 56 (1):256–62.

Wakeland, Anna K., Francesca Soncin, Matteo Moretto-Zita, Ching-Wen Chang, Mariko Horii, Don Pizzo, Katharine K. Nelson, Louise C. Laurent, and Mana M. Parast. 2017. "Hypoxia Directs Human Extravillous Trophoblast Differentiation in a Hypoxia-Inducible Factor-Dependent Manner." *The American Journal of Pathology* 187 (4):767–80. https://doi.org/10.1016/j.ajpath.2016.11.018.

Waldenström, Urban, Ann-Britt Engström, Dan Hellberg, and Staffan Nilsson. 2009. "Low-Oxygen Compared with High-Oxygen Atmosphere in Blastocyst Culture, a Prospective Randomized Study." *Fertility and Sterility* 91 (6):2461–65. https://doi.org/10.1016/j.fertnstert.2008.03.051.

Wale, Petra L., and David K. Gardner. 2016. "The Effects of Chemical and Physical Factors on Mammalian Embryo Culture and Their Importance for the Practice of Assisted Human

Reproduction." *Human Reproduction Update* 22 (1):2–22. https://doi.org/10.1093/humupd/dmv034.

Wang, Jeff, and Mark V Sauer. 2006. "In Vitro Fertilization (IVF): A Review of 3 Decades of Clinical Innovation and Technological Advancement." *Therapeutics and Clinical Risk Management* 2 (4):355–64.

Wang, Lingjuan, Xiaoyan Xu, Lina Kang, and Wenpei Xiang. 2016. "Bone Marrow Mesenchymal Stem Cells Attenuate Mitochondria Damage Induced by Hypoxia in Mouse Trophoblasts." *PloS One* 11 (4):e0153729. https://doi.org/10.1371/journal.pone.0153729.

Wang, Yuping, and Shuang Zhao. 2010. *Vasoactivators and Placental Vasoactivity*. Morgan & Claypool Life Sciences. https://www.ncbi.nlm.nih.gov/books/NBK53257/.

Watson, A. J. 1992. "The Cell Biology of Blastocyst Development." *Molecular Reproduction and Development* 33 (4):492–504. https://doi.org/10.1002/mrd.1080330417.

Watson, A. J., and L.C. Barcroft. 2001. "Regulation of Blasotcyst Formation." *Frontiers in Bioscience: A Journal and Virtual Library* 6 (May):708–30.

Weenen, Christien, Joop S. E. Laven, Anne R. M. Von Bergh, Mark Cranfield, Nigel P. Groome, Jenny A. Visser, Piet Kramer, Bart C. J. M. Fauser, and Axel P. N. Themmen. 2004. "Anti-Müllerian Hormone Expression Pattern in the Human Ovary: Potential Implications for Initial and Cyclic Follicle Recruitment." *Molecular Human Reproduction* 10 (2):77–83.

Wells, Dagan, Kulvinder Kaur, Jamie Grifo, Michael Glassner, Jenny C Taylor, Elpida Fragouli, and Santiago Munne. 2014. "Clinical Utilisation of a Rapid Low-Pass Whole Genome Sequencing Technique for the Diagnosis of Aneuploidy in Human Embryos Prior to Implantation." *Journal of Medical Genetics* 51 (8):553–62. https://doi.org/10.1136/jmedgenet-2014-102497.

Wheeler, M. B., and M. Rubessa. 2016. "Integration of Microfluidics and Mammalian IVF." *Molecular Human Reproduction*, July. https://doi.org/10.1093/molehr/gaw048.

Wiley, L. M., S. Yamami, and D. Van Muyden. 1986. "Effect of Potassium Concentration, Type of Protein Supplement, and Embryo Density on Mouse Preimplantation Development in Vitro." *Fertility and Sterility* 45 (1):111–19.

Wilson, J. D., F. W. George, and J. E. Griffin. 1981. "The Hormonal Control of Sexual Development." *Science (New York, N.Y.)* 211 (4488):1278–84.

Winslow, Russell. 2017. *Organism and Environment: Inheritance and Subjectivity in the Life Sciences*. Lexington Books.

Yang, Yu, Marcia Arenas-Hernandez, Nardhy Gomez-Lopez, Jing Dai, Graham C. Parker, Elizabeth E. Puscheck, and Daniel A. Rappolee. 2016. "Hypoxic Stress Forces

Irreversible Differentiation of a Majority of Mouse Trophoblast Stem Cells Despite FGF4." *Biology of Reproduction* 95 (5):110. https://doi.org/10.1095/biolreprod.116.138412.

Yang, Yu, Alan Bolnick, Alexandra Shamir, Mohammed Abdulhasan, Quanwen Li, G. C. Parker, Elizabeth E. Puscheck, and D. A. Rappolee. 2017. "Blastocyst-Derived Stem Cell Populations under Stress: Impact of Nutrition and Metabolism on Stem Cell Potency Loss and Miscarriage." *Stem Cell Reviews* 13 (4):454–64. https://doi.org/10.1007/s12015-017-9734-4.

Yang, Yu, Yanwen Xu, Chenhui Ding, Rabea Youcef Khoudja, Mingmei Lin, Awoniyi O. Awonuga, Jing Dai, Elizabeth E. Puscheck, Daniel A. Rappolee, and Canquan Zhou. 2016. "Comparison of 2, 5, and 20 % O2 on the Development of Post-Thaw Human Embryos." *Journal of Assisted Reproduction and Genetics*, March. https://doi.org/10.1007/s10815-016-0693-5.

Yang, Zhihong, James Lin, John Zhang, Wai Ieng Fong, Pei Li, Rong Zhao, Xiaohong Liu, William Podevin, Yanping Kuang, and Jiaen Liu. 2015. "Randomized Comparison of Next-Generation Sequencing and Array Comparative Genomic Hybridization for Preimplantation Genetic Screening: A Pilot Study." *BMC Medical Genomics* 8 (1). https://doi.org/10.1186/s12920-015-0110-4.

Yang, Zhihong, Shala A Salem, Xiaohong Liu, Yanping Kuang, Rifaat D Salem, and Jiaen Liu. 2013. "Selection of Euploid Blastocysts for Cryopreservation with Array Comparative Genomic Hybridization (ACGH) Results in Increased Implantation Rates in Subsequent Frozen and Thawed Embryo Transfer Cycles." *Molecular Cytogenetics* 6 (August):32. https://doi.org/10.1186/1755-8166-6-32.

Yap, Alpha S., Guillermo A. Gomez, and Robert G. Parton. 2015. "Adherens Junctions Revisualized: Organizing Cadherins as Nanoassemblies." *Developmental Cell* 35 (1):12–20. https://doi.org/10.1016/j.devcel.2015.09.012.

Zaidel-Bar, Ronen. 2013. "Cadherin Adhesome at a Glance." *J Cell Sci* 126 (2):373–78. https://doi.org/10.1242/jcs.111559.

Zartman, Jeremiah J., Jitendra S. Kanodia, Nir Yakoby, Xenia Schafer, Colin Watson, Karin Schlichting, Christian Dahmann, and Stanislav Y. Shvartsman. 2009. "Expression Patterns of Cadherin Genes in Drosophila Oogenesis." *Gene Expression Patterns: GEP* 9 (1):31–36. https://doi.org/10.1016/j.gep.2008.09.001.

Zhao, Jing, Qiong Zhang, and Yanping Li. 2012. "The Effect of Endometrial Thickness and Pattern Measured by Ultrasonography on Pregnancy Outcomes during IVF-ET Cycles." *Reproductive Biology and Endocrinology* 10:100. https://doi.org/10.1186/1477-7827-10-100.

Zhou, Jianjun, Bin Wang, Yali Hu, and Haixiang Sun. 2017. "Association between the Number of Oocytes Retrieved and Cumulative Live Birth Rate in Women Aged 35-40 Years

Undergoing Long GnRH Agonist IVF/ICSI Cycles." *Archives of Gynecology and Obstetrics*, September. https://doi.org/10.1007/s00404-017-4503-9.

Zhu, Hai Bo, Zhi Hong Zhang, Elfateh Fadlalla, Rui Xue Wang, Dong Feng Geng, and Rui Zhi Liu. 2014. "Culturing Surplus Poor-Quality Embryos to Blastocyst Stage Have Positive Predictive Value of Clinical Pregnancy Rate." *Iranian Journal of Reproductive Medicine* 12 (9):609–16.

Zhu, Ruifang, Xiangyu Zhu, Ying Yang, Honglei Duan, Ying Zhang, Xing Wu, Wanjun Wang, and Jie Li. 2014. "Application of different technologies for distinguishing true and pseudo mosaicisms during prenatal diagnosis." *Zhonghua Yi Xue Yi Chuan Xue Za Zhi = Zhonghua Yixue Yichuanxue Zazhi = Chinese Journal of Medical Genetics* 31 (5):636–40. https://doi.org/10.3760/cma.j.issn.1003-9406.2014.01.022.

Zohni, Khaled M., Itai Gat, and Clifford Librach. 2016. "Recurrent Implantation Failure: A Comprehensive Review." *Minerva Ginecologica*, March.